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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of Gautvik et al..	:	
	:	Group Art Unit: 1812
Serial No. 08/340,664	:	
	:	Examiner: L. Spector
Filed: November 16, 1994	:	
	:	
For: PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS	:	
	X	

Assistant Commissioner for Patents
Washington, D. C. 20231

DECLARATION OF JOHN E. MAGGIO, Ph.D. PURSUANT TO 37 C.F.R. § 1.132

Sir:

I, John E. Maggio, declare as follows:

1. I am a citizen of the United States of America residing at 480 Washington Street, Brookline, Mass. 02146.

2. I am an Associate Professor of Biological Chemistry and Molecular Pharmacology at Harvard Medical School, Boston, Massachusetts. I have been a professor at Harvard Medical School since 1985.

3. My formal education includes Bachelors, Masters and Doctoral degrees, in the fields of chemistry and organic chemistry, all from Harvard University. I have also had extensive post-doctoral training at the University Chemical Laboratory and MRC Neurochemical Pharmacology Unit, Cambridge University, Cambridge, England, and at the Neuropsychopharmacology Research Unit at Yale University Medical School in New Haven. Both post-doctoral positions involved protein and peptide chemistry and purification thereof. My current curriculum vitae, including a list of my publications is attached as Exhibit 1.

4. My past and present work centers around synthesis, purification and characterization of biologically active peptides including tachykinins, magainins and amyloid peptides. As part of my work and since at least the late 1970s, I have used various forms of chromatography and electrophoresis for the purification of peptides and for their characterization. I am fully familiar with these techniques, and the state of their development throughout the 1980s and 1990s. I am also fully familiar with the past and present capabilities and limitations of such techniques. Representative of my work with peptide separation and characterization is an article attached as Exhibit 2 entitled "Mapping Peptide-binding Domains of the Substance P(NK-1) Receptor from P388D_1 Cells with Photolabile Agonists", *J. Biological Chemistry*. 270, (1995), 1213-1220.

5. My knowledge of chromatography, electrophoresis, and other techniques used commonly in protein chemistry stems from my repeated use of those techniques throughout my career. I have supervised students and other scientists using these techniques and have taught the techniques, both in the classroom and in the laboratory. Therefore, I am comfortable judging the ordinary level of skill that a person in this art would possess in terms of the theoretical and the bench aspects of these techniques.

6. In preparing this declaration I reviewed, among other things, the following materials: a copy of the specification of U.S. Serial No. 08/340,664, filed November 16, 1994, (the "'664 application"); a copy of the Official Action dated September 8, 1995 issued by the patent examiner, Dr. Spector; copies of each of the references identified in the

Official Action; and a copy of the Declaration of Kaare M. Gautvik, M.D. Pursuant to 37 C.F.R. § 1.132 as well as the documents and photographs attached thereto.

7. I understand from my review of the Official Action, the United States Patent and Trademark Office has refused to grant the '664 application in view of the disclosures of four references: *Brewer et al.*, *Fairwell et al.*, *Kimura et al.* and/or *Kumagaye et al.*

8. I have reviewed the four references cited by the Patent Office and I do not agree with the Patent Office's conclusions regarding their teachings or disclosures. In my opinion, none of the references describe or suggest a method of obtaining a substantially pure, intact, hPTH peptide. None of the references provides a basis for concluding that a substantially pure hPTH product was actually produced. Further, nothing in the references describes an hPTH peptide having biological activity substantially equivalent to naturally occurring hPTH. I believe that a biochemist, organic chemist or analytical chemist having an ordinary level of skill in this technology would not be unable to draw any conclusion with regard to the purity of hPTH produced in accordance with the cited references. If anything, given the errors appearing in those references and the known shortcomings of the techniques described in the references, *e.g.* solid phase chemical synthesis, those of ordinary skill in the art would probably assume that the resulting hPTH material was impure.

9. *Brewer et al.* relate to an isolation from tissue, not a recombinant material. *Brewer et al.* contain three errors at positions 22, 28 and 30 of the synthesized peptide compared to the wild-type peptide. This is illustrated

in Fig. 1 of *Brewer et al.* Accordingly, *Brewer et al.* do not teach the production of an *intact* hPTH peptide. Moreover, two later publications cited by Dr. Spector in the Official Action, namely *Kimura et al.* and *Kumagaye et al.*, show that the purification protocols discussed in *Brewer et al.* result in impure materials. For example, Fig. 2, on page 496 of *Kimura et al.* is an HPLC profile of crude product obtained after use of a separation protocol analogous to that disclosed in *Brewer et al.*; namely, the use of a combination of gel filtration and ion exchange chromatography. Impurities are plainly evident. Therefore, a conclusion of homogeneity based on *Brewer et al.* is unjustified. Further, *Kimura et al.* describe a purification sequence of CM-cellulose column chromatography followed by gel filtration on Sephadex G-50, followed in turn by the use of reverse phase-high pressure liquid chromatography ("RP-HPLC"). *Kimura et al.* added the RP-HPLC step in recognition of the need to obtain better purity than *Brewer et al.* obtained. This fact alone, in my opinion, eliminates any plausible basis for concluding that the protein resulting from the methods described in *Brewer et al.* was essentially pure.

10. Many of the criticisms of *Brewer et al.* apply to *Fairwell et al.* For example, *Fairwell et al.* produced a peptide having an Asp at position 76. Native hPTH has an Asn in that position. *Fairwell et al.* also used a separation protocol combining the use of gel filtration and ion exchange chromatography. As previously mentioned, that protocol was re-run by *Kimura et al.* and the results, as illustrated in the chromatogram in Fig. 2 thereof, show significant impurities. Finally, as *Kimura et al.* used RP-HPLC rather than relying

merely on the separation protocol described in *Fairwell et al.*, it is clear that subsequent investigators believed that the separation protocols of *Fairwell et al.* were inadequate.

11. *Kimura et al.* did not produce an essentially pure hPTH. As *Kumagaye et al.* clearly explain, "[t]oday, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system; the present results clearly indicate that the purification of synthetic peptides by RP-HPLC is not sufficient to obtain homogeneous products." (Emphasis added) *Kumagaye et al.* at page 330. This is especially significant because *Kumagaye et al.* is the same group of researchers as *Kimura et al.*

12. *Kumagaye et al.* disclose a method of separating two different forms of hPTH from a mixture thereof by using cation exchange-HPLC. This is not a particularly surprising result as the two forms of hPTH disclosed have a full charge difference between them, a situation ideal for the use of cation exchange-HPLC. Nonetheless, one of ordinary skill in the art would not conclude that the resulting hPTH in accordance with *Kumagaye et al.* was essentially pure. One could conclude that the resulting material was pure of the one specific impurity, i.e. the specific point mutated form disclosed. However, there is no basis for concluding further. If anything, as explained herein, there is every reason to believe that impurities are present.

13. *Kumagaye et al.* describe a solid phase peptide synthesis protocol which was common at the time. That synthesis, as explained in the *Kimura et al.* article, involved the use of BOC protected amino acids and traditional BOC chemistry. Using BOC chemistry, each successive amino acid is

added to the N-terminus of a growing chain by first removing the blocking group in acid, then neutralizing prior to coupling, followed by coupling the next BOC amino acid in sequence using, for example, dicyclohexylcarbodiimide ("DCC").

14. This technology suffers from a number of well known shortcomings and, in fact, has largely been replaced. One of the better known and most common problems with solid phase BOC chemistry, particularly for longer peptides such as hPTH, is racemization. As explained in *Bodanszky,* "Peptide Chemistry; A Practical Textbook" at page 120, the problem of racemization using DCC coupling and BOC chemistry is well documented. *See* Exhibit 3. This chapter, as well as the others attached as Exhibit 3, demonstrate the prevalence of racemization and concerns over this phenomenon during solid phase protein synthesis.

15. Some of the other well known impurities generated by solid phase synthesis are described in the *Fairwell et al.* article cited by Dr. Spector at page 2691. These impurities include, among other things, deletion peptides, omission peptides and prematurely terminated peptides. For example, during solid phase synthesis it is possible for coupling to be either duplicative or incomplete, thereby providing a peptide having an additional amino acid or an omission from the normal sequence. There may be one or more additions and/or deletions in any given peptide. These additions and/or deletions can occur almost anywhere along the chain. Premature termination of the chain length is also common. This may occur for a host of reasons such as, peptides folding in on themselves, side reactions to make the N-terminal amino acid unavailable for

further coupling, steric hindrance, premature cleavage from the bead, and the like.

16. To fully understand the magnitude of the purification problems presented by the use of this type of synthetic chemistry, one needs to consider that all three of the foregoing problems, incomplete coupling, premature termination, and racemization, are occurring simultaneously. The result often is a wide variety of incorrect peptides, frequently including two or more of the aforementioned errors. The frequency of these errors, and therefore the degree of impurity, increases exponentially with the length of desired peptide. Proteins such as hPTH, which is 84 amino acids in length, are considered to be long and difficult to make synthetically, even by today's sophisticated standards. The technique employed by *Kumagaye et al*, cation exchange-HPLC, could be used for separating some of the resulting impurities. However, this technique would only work for that fraction of the total impurities having a charge differential when compared to native hPTH; a relatively minor percentage of the total impurities. In addition, depending upon the conditions used, not all of the differently charged species will be separated. Some of the resulting impurities may have a charge which is very similar to native hPTH, and may co-elute with hPTH. Any single impurity, if known, could theoretically be removed from the mixture by HPLC, RP-HPLC, and/or some other separation technique(s). Here, with the many possible impurities, it would be nearly impossible to effectively remove them all.

17. Persons familiar with cation exchange-HPLC would realize that the types of impurities to which I have referred result, almost inevitably, from the use of solid phase BOC

chemistry and that many of the impurities co-elute with intact hPTH. Therefore, one would conclude, as I concluded, that the hPTH material resulting from the protocol described in *Kumagaye et al.* is pure only insofar as the one disclosed point mutant. No further conclusions about purity can be made. If anything, the impurities known to result from the solid phase synthesis described in the references would lead to the conclusion that the hPTH resulting from *Kumagaye et al.* would contain other impurities. *Kumagaye et al.* provide no explicit recitations of purity, provide no other form of characterization of the quality or quantity of the resulting hPTH material and provide nothing with regard to biological activity. Due to the cellular editing mechanisms found in, for example, yeast and *E. coli*, such impurities would not occur.

18. My opinion of all of the references, and, in particular *Kumagaye et al.*, is strengthened by the comparisons that I have reviewed between recombinant hPTH manufactured as described in the '664 application and commercially available, synthetically produced, hPTH produced by solid phase peptide synthesis.

19. As stated in paragraph 6, I have reviewed the declaration of Dr. Kaare M. Gautvik and, in particular, the photographs labeled Glossy 0 through Glossy III attached in Exhibits B-E, respectively, thereto. I understand from Dr. Gautvik's declaration that the materials analyzed and depicted in these photographs were made pursuant to the techniques described in the '664 application. Having reviewed that specification, I have no reason to question that assertion. The photographs are particularly informative because they provide a direct comparison between peptides produced by solid

phase chemical synthesis and recombinant technology as described in the '664 application.

20. Glossy 0 illustrates an electrophoretic gel comparing recombinantly produced hPTH from Dr. Gautvik's laboratory with hPTH produced by solid phase synthesis sold by Sigma. Lane 2 (second from the left) contains the recombinant hPTH produced by Dr. Gautvik. The single broad band indicates homogeneity. In contrast, the Sigma material illustrated in Lane 3 shows a band migrating at roughly the same position as the hPTH produced recombinantly and two additional impurities of higher molecular weight. Based on the presence of these impurities in the Sigma material, the intensity and breadth of the bands and the relative intensities and sizes of the bands of hPTH, it is not hard to see that the recombinant material is orders of magnitude purer than the Sigma material.

21. Glossy III shows molecular weight standards in Lane S as well as recombinant hPTH produced from *E. coli* (Lane 4) and yeast (Lane 2) produced in accordance with the procedures outlined in the '664 application. Disposed between these materials, in Lane 3 is a synthetic material produced by solid phase synthesis available from a second chemical supplier, Bachem. The recombinant material is characterized by a single, sharp, dark, broad band corresponding to hPTH. In contrast, the Bachem lane illustrates the presence of lower molecular weight impurities in a smear. Moreover, the difference in the intensity of the staining indicates a significantly greater amount of hPTH in the recombinant preparations than in the chemically synthesized preparations, using an identical load (800 nanograms) of assayed material.

22. Glossy II contains, in addition to the information illustrated in Glossy III (lanes 27 through 29), identical preparations at a loading of 200 nanograms, (lanes 22 through 24). The difference in the intensities of the bands between the recombinantly produced material and the solid phase synthetic material available from Bachem illustrates the significantly greater amount of hPTH in the recombinant material, per unit weight. (Lanes 22 and 24 contain recombinant material and line 23 contains synthetic.) This information is totally consistent with the HPLC, N-terminal amino acid sequencing, mass spectrometry and two dimensional gel electrophoresis described in the '664 application. Based on this information, one of any level of skill in this art would conclude, as I have, that the recombinant material produced in accordance with the present invention is essentially pure.

23. My opinions are both verified and amplified by my review of Dr. Gautvik's declaration and, in particular, his publication in the peer-refereed journal *Peptides,* attached to his Declaration as Exhibit F. This article clearly demonstrates the biological properties of the recombinant material produced in accordance with the '664 application and verifies that which would be implicitly understood therefrom; namely that the hPTH material of the invention has biological activity substantially equivalent to naturally occurring human parathyroid hormone. Chemically synthesized material does not.

24. Dr. Gautvik's *Peptides* article is significant in that it illustrates both *in vivo* and *in vitro* biological activity. The results consistently reemphasize the superiority of the recombinant hPTH material in direct side-by-side comparisons to synthetic material. Fig. 1 of the *Peptides*

paper illustrates the differences in binding affinity between recombinant hPTH produced in accordance with the '664 application from both *E. coli* and yeast and synthetic material from Bachem. The K_d of the recombinant material was 9.5nM while the K_d of the Bachem material was 18nM. This illustrates that the recombinant material contains approximately twice as much authentic hPTH when compared to the chemically synthesized material. The differences between these K_d values are very statistically significant as described in the paper (95% confidence intervals and redundant testing in triplicate). Because the K_d values indicate a greater amount of authentic hPTH per unit weight, the significant difference between the two K_d values indicate a dramatic difference in purity.

25. Fig. 2 of the *Peptides* paper illustrates the abilities of different preparations of hPTH to elicit a biological response in cell cultures. From Fig. 2 one can determine both an EC_{50} for cyclic AMP (cAMP) as well as a measure of efficacy or maximal response. EC_{50} is a measure of the potency of the materials in question. Here, the EC_{50} for recombinant hPTH is 1.5nM. The Ec_{50} for the Bachem material was 5.7nM. This is almost a four fold difference. As the figure and the accompanying text illustrate, this difference is highly statistically significant.

26. One of the more surprising findings outlined in the *Peptides* paper is the efficacy of the resulting materials. It appears that the Bachem material is only about 70% as efficacious as the recombinant hPTH. This means that no dose of synthetic material would be able to produce the maximal response of the tested cells, a problem not shared by the recombinant material. These two figures, acting in

combination, illustrate that the recombinant material is superior not only in purity, but also in binding and in eliciting a biological response. Moreover, the data just described are completely consistent with the data illustrated in Figs. 3-5 which show the *in vivo* activity of recombined hPTH in rats. In particular, Fig. 3 confirms the efficacy and potency data described in Fig. 2 by exhibiting an increased level of blood calcium over that achievable through the use of the synthetic material. Even 2.7 micrograms of Bachem PTH was unable to produce the same results as 2.0 micrograms of recombinant PTH.

27. The *Peptide* paper is a particularly good comparison of the recombinant material produced in accordance with the '664 application and synthetically produced material because of its careful characterizations and the variety of analytical techniques used. For example, peptide concentrations were determined by amino acid analysis. This is the premier method of determining peptide concentrations to date and is superior to other techniques such as optical density or dry weight. *In vivo* activity was measured not in one system, but rather by induction of hypercalcemia, urinary excretion of phosphate and by changes in urinary cAMP after administration of hPTH. Moreover, *in vitro* activity was assayed by receptor binding and cAMP responses of cells in culture. The variety of techniques used would appear to conclusively establish the superiority of recombinant material over synthetic material, both biologically and in terms of its purity. For these reasons, I believe that essentially pure recombinant material results from the practice of the invention described in the above-captioned application and that this hPTH

material is superior to anything in the prior art. I also believe that one of ordinary skill in the art would, upon reading the application, conclude as I have.

28. I have been warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon. I declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date: 6 MARCH 1996

JOHN E. MAGGIO, Ph.D.

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Exh 1

CURRICULUM VITAE

NAME John E. Maggio

ADDRESS: Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School
240 Longwood Avenue
Boston, MA 02115

DATE OF BIRTH: November 6, 1952

PLACE OF BIRTH: Houston, Texas

EDUCATION:

1975	A.B.	Harvard College, Cambridge, MA (Chemistry)
1975	A.M.	Harvard University, Cambridge, MA (Chemistry)
1981	Ph.D.	Harvard University, Cambridge, MA (Organic Chemistry)

POSTDOCTORAL TRAINING:

1981-83	Postdoctoral Research Associate, University Chemical Laboratory and MRC Neurochemical Pharmacology Unit, Cambridge, England
1984-85	Postdoctoral Fellow, Neuropsychopharmacology Research Unit, Yale University School of Medicine, New Haven, CT

ACADEMIC APPOINTMENTS:

1985-87	Assistant Professor of Pharmacology, Harvard Medical School, Boston, MA
1987-91	Assistant Professor of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA
1991-	Associate Professor of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

AWARDS AND HONORS:

1971-75	Dean's List, Harvard College Scholarship for Outstanding Academic Achievement, National Merit Scholar
1975	A.B. *magna cum laude* with Highest Honors
1976-78	National Science Foundation Graduate Fellow
1981-82	Member of the High Table, King's College, Cambridge, England
1981-82	North Atlantic Treaty Organization / National Science Foundation Postdoctoral Fellow
1983-84	Muscular Dystrophy Association Postdoctoral Fellow

ORIGINAL PUBLICATIONS:

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AFFILIATIONS:

American Chemical Society
Society for Neuroscience
International Brain Research Organization
American Association for the Advancement of Science
Foundation for Biomedical Research
American Peptide Society
International Neuropeptide Society
Boston Area Neuroscience Group

COMPETITIVE SUPPORT:

Science and Engineering Research Council, UK (1981-82)
National Science Foundation (1982)
Muscular Dystrophy Association (1983-84)
North Atlantic Treaty Organization (1982)
National Institute of Neurological Disorders & Stroke (1985-1994)
Milton Fund of Harvard University (1989) (1994)
National Institute of General Medical Sciences (1988-1993)(1995-date, *ACTIVE*)
Institute of Chemistry in Medicine/Hoffmann-La Roche (1992-date, *ACTIVE*)
American Health Assistance Foundation (1994-date, *ACTIVE*)
National Institute of Aging (1995-date, *ACTIVE*)

TRAINING PROGRAMS (NIH) at Harvard Medical School:

Pharmacological Sciences (Associate)
Endocrinology (Associate, Executive Committee)
Neuroscience (Associate, Admissions, Appointments, Steering Committee)
Developmental Neurology (Associate)
Molecular Biophysics (Associate)
Neurological Sciences Academic Development (Associate)
Biological Sciences in Public Health (Associate)

LOCAL COMMITTEES:

Dr. Maggio has served on and chaired a wide range of departmental, program, medical school, and university committees, such as: Faculty Search, Admissions, Facilities, Thesis Advisory, Qualifying/Preliminary Examination, Prize, Curriculum, Thesis Defense, Course Planning, Steering, Executive, Criteria, Etc. *See Service.*

TEACHING EXPERIENCE:

Mathematics (undergraduate)
Organic Chemistry (undergraduate)
Biochemistry (undergraduate, graduate, medical)*
Neuropharmacology (graduate, medical)*
Membranes, Receptors and Signal Transduction (graduate)*
Pharmacology (graduate, medical)
Biological Chemistry and Molecular Pharmacology (graduate)*
Conduct of Science (graduate)
Membrane Structure & Function (graduate)*
*Course Director or Co-Director

PEER REVIEW EXPERIENCE:

NIH Study Sections (ad hoc, Reviewers Reserve):
Experimental Cardiovascular Sciences (ECS, 4/87)
Small Business Innovation Research (SSS-7/E, 3/88)
AIDS and Related Research (ARR-5, 12/88)
Neurological Sciences (NLS-1, 6/90, 10/90, 6/91, 10/91, 10/92, 10/93, 10/94)
Neurology (NEUB-1, 6/95)

NSF Applications (ad hoc)

Journals (ad hoc):
Am. J. Pathol., Am. J. Physiol., Anal. Biochem., Anesthesiology, Biochemistry, Biochem. Biophys. Acta, Brain Res., Cancer Res., FEBS Lett., Gastroenterology, J. Biol. Chem., J. Chem. Neuroanat., J. Lab. Clin. Med., J. Neurochem., J. Neuroimmunol., J. Neurosci., Lab. Invest., Nature, Neurobiol. Aging, Peptides, Pharmacol. Rev., Proc. Natl. Acad. Sci. USA, Protein Sci., Regul. Peptides, Trends Neurosci.

BRIEF PROFESSIONAL BIOGRAPHY:

Dr. Maggio's graduate work included research on noncovalent interactions, organic synthesis, reaction mechanisms, biological control, NMR spectroscopy, and biochemistry under the mentorship of Jean-Marie Lehn, Robert B. Woodward, and (principally) Konrad E. Bloch. He received the Ph.D. in Organic Chemistry from Harvard University in 1981. His postdoctoral research on various aspects of neuropeptides and neuropeptide receptors was carried out at the Medical Research Council and the University of Cambridge, UK, with Leslie L. Iversen and Dudley H. Williams; and later at Yale University School of Medicine with Robert H. Roth. He joined the faculty of Harvard Medical School in 1985, and is presently Associate Professor of Biological Chemistry and Molecular Pharmacology.

RESEARCH INTERESTS

The bioactive peptides are the largest and least understood class of intercellular messengers, carrying out a diverse set of functions in a wide variety of systems. Understanding bioactive peptides and their receptors, in the nervous system and elsewhere, is the general research goal in our group.

One system of interest is the tachykinin (substance P) family of peptides and receptors, which are involved in transmission of primary afferents and thus in pain and neurogenic inflammation. As the primary structures of both the ligands and their receptors are known, an excellent model system for peptide-protein interactions in signalling is available. Recently we have identified through photoaffinity labelling which regions of the peptide substance P interact with which regions of its G-protein-coupled receptor, a protein whose expression is upregulated a thousand-fold in some inflammatory diseases. Radioactive, fluorescent, and antibody probes of these receptors allow studies of desensitization and internalization *in vivo* and *in vitro*.

Another system under investigation is the process of amyloid formation in Alzheimer's disease (AD) and other amyloidoses. The characteristic lesion of AD is brain senile plaques formed mainly of the human amyloid peptide Aß, a ≈40-mer which occurs naturally in normal as well as AD brain. By reconstituting plaque growth (deposition of Aß at physiological concentrations onto authentic plaques) *in vitro*, we can characterize the process and identify conditions and components which enhance or inhibit its kinetics. Structure/activity studies have identified amino acids critical for amyloid deposition and active peptide analogues suitable for high resolution structure determination by nuclear magnetic resonance spectroscopy. The latter studies have further identified conformational elements essential to plaque deposition.

Another interest is the characterization of novel bioactive peptides from natural sources. A particularly rich source is the skin venom of certain neotropical frogs. The peptides found here include antibiotics and toxins as well as close analogs of discovered and yet undiscovered mammalian neuropeptides.

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Mapping Peptide-binding Domains of the Substance P (NK-1) Receptor from P388D$_1$ Cells with Photolabile Agonists*

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Yue-Ming Li, Margarita Marnerakis, Evelyn R. Stimson, and John E. Maggio‡

From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

The tachykinin substance P (SP) is a peptide transmitter of primary afferents. Its actions on both central and peripheral targets are mediated by a G-protein-coupled receptor of known primary structure. To identify contact sites between the undecapeptide SP and its receptor, we prepared radiolabeled photoreactive analogs of SP (H-RPKPQQFFGLM-NH$_2$) by replacing amino acids in the peptide with *p*-benzoyl-L-phenylalanine (BPA). SP, BPA^3-SP, and BPA^8-SP bind with high affinity (K_d < 3 nM) to SP receptors on the murine cell line P388D$_1$, triggering intracellular calcium responses. Both binding and calcium responses are blocked by the specific SP receptor antagonist CP-96345. On photolysis, radioiodinated BPA^3-SP and BPA^8-SP covalently label a heterogeneously glycosylated protein of about 75 kDa; labeling is abolished by excess unlabeled SP or CP-96345. The labeled receptors were digested with V8 protease and/or trypsin, and the resulting fragments were analyzed by electrophoresis, high pressure liquid chromatography, and chemical or enzymatic modification. BPA^3-SP and BPA^8-SP photoincorporate into different regions of the murine SP receptor. The results establish that the third and the eighth positions of SP, respectively, interact with the NH$_2$-terminal extracellular tail (residues 1–21) and second extracellular loop (residues 173–183) of the SP receptor. A model for the agonist peptide-binding sites of the SP receptor is proposed based on photoaffinity labeling and mutagenesis studies.

A large majority of the known receptors belong to the G-protein-coupled receptor superfamily (Baldwin, 1994). These receptors are characterized by the presence of seven hydrophobic regions of primary structure thought to represent transmembrane domains. The receptors lie in the bilayer such that the amino-terminal region of the protein is extracellular and the carboxyl-terminal region is cytoplasmic. The agonists which bind to and activate G-protein-coupled receptors vary widely in size, from glycoprotein hormones (>30 kDa) to single photons. The larger agonists (>10 kDa; *e.g.* thyroid stimulating hormone and follicle stimulating hormone) bind to the amino-terminal region of their G-protein-coupled receptors, while the smaller agonists (<0.2 kDa; *e.g.* norepinephrine, serotonin, and photons) bind within the plane of the bilayer between the seven transmembrane domains (Bockaert, 1991). Essentially all characterized receptors for bioactive peptides (0.5–5 kDa) are also members of the G-protein-coupled receptor superfamily, but which regions of their receptors interact with these agonists of intermediate size has not yet been defined.

The undecapeptide substance P (SP)[1] has been identified as a neurotransmitter associated with pain modulation and neurogenic inflammation (Pernow, 1983; Otsuka and Yoshioka, 1993). SP belongs to the tachykinin peptide family which is characterized by a conserved COOH-terminal sequence -FXGLM-NH$_2$, where *X* is an aromatic or aliphatic amino acid (Maggio, 1988). The SP receptor (also known as the neurokinin-1 or NK-1 receptor) has been cloned from several species including human, mouse, rat, and guinea pig and displays a very high degree of primary sequence homology across species (Gerard *et al.*, 1993). The SP receptor (SPR) is a member of the G-protein-coupled receptor superfamily, as are receptors for other peptides in the tachykinin family.

Chimeric and point-mutated SP receptors have been constructed to probe receptor structure-function in an attempt to identify binding domains for peptide agonists and nonpeptide antagonists as well as domains associated with agonist-stimulated second messenger responses (*e.g.* Cascieri *et al.*, 1994; Fong *et al.*, 1992a, 1992b, 1993, 1994a 1994b; Gether *et al.*, 1993a, 1993b, 1993c, 1994; Huang *et al.*, 1994a, 1994b; Jensen *et al.*, 1994; Sachais *et al.*, 1993; Yokota *et al.*, 1992; Zoffmann *et al.*, 1993). These studies have indicated that both the extracellular and transmembrane domains of the SP receptor are important for the binding of agonist, and several specific residues conserved in all species examined have been identified as important for peptide binding. Analysis of SP analogs further suggested the COOH-terminal carboxyamide of SP may interact with residues in the second transmembrane domain (Huang *et al.*, 1994b). However, the identification of a particular residue as necessary for agonist binding does not necessarily imply direct interaction of that side chain with agonist, as loss of function may instead result from changes in protein folding. Since the binding of SP (1350 Da) must involve a larger number of receptor/ligand contacts than small nonpeptide agonists (*e.g.* norepinephrine, 170 Da), it has not been possible to define the interaction of SP and its receptor by mutagenesis alone.

Photoaffinity labeling has been proven to be a useful tool in identifying structural domains of receptors involved in ligand

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‡ To whom correspondence should be addressed: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115. Tel.: 617-432-0757; Fax: 617-432-3833.

[1] The abbreviations used are: SP, substance P; BPA, *p*-benzoyl-L-phenylalanine; BPA^3-SP, Y^{0}BPA^3-SP; BPA^8-SP, Y^{0}BPA^8-SP; CP-96345, (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-*methyl*]-1-aza-bicyclo[2.2.2]octan-3-amine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); Endo F, *F. meningosepticum* endoglycosidase F; FCS, fetal calf serum; Fmoc, fluoren-9-ylmethoxycarbonyl; HPLC, high performance liquid chromatography; NK-1R, neurokinin-1 receptor (same as SPR); PAGE, polyacrylamide gel electrophoresis; SPR, substance P receptor (same as NK-1R); TM, transmembrane; V8, *S. aureus* V8 protease; cpm, counts/minute.

binding (*e.g.* Dohlman *et al.*, 1991). This technique offers a unique approach by directly identifying the contact regions of a receptor and its ligands. As an essential complement to mutagenesis approaches, we have applied photoaffinity labeling to identify agonist peptide binding domains of the SP receptor. *p*-Benzoyl-L-phenylalanine (BPA), a photoreactive amino acid, has been used to replace amino acids in peptides for receptor photoaffinity labeling (Dorman and Prestwich, 1994). Photoactivated (triplet biradical) BPA reacts preferentially with C-H bonds but has low reactivity toward water; furthermore, the chromophore can be activated in the visible, avoiding protein-damaging UV wavelengths. In previous work by others (Boyd *et al.*, 1991a, 1991b, 1994; Kage *et al.*, 1993), an SP derivative containing BPA at position 8 and acylated with 3-(3-iodo-4-hydroxyphenyl) propionic acid at the side chain of Lys^3 has been synthesized to study the SP receptor. Photolysis of this ligand with membrane-bound SP receptors from rat submaxillary gland led to about 70% incorporation of bound label into two polypeptides (46 and 53 kDa); enzymatic studies suggested that the smaller protein resulted from proteolysis of the larger (Kage *et al.*, 1993).

$P388D_1$ cells, a nontransfected murine macrophage/monocyte cell line (Dawe and Potter, 1957), express a high density of functional SP receptors (Persico *et al.*, 1988; Li *et al.*, 1994) but no detectable levels of other tachykinin receptors.[2] The SP receptors of this cell line are coupled to Ca^{2+} mobilization (Li *et al.*, 1994). In the present study we have used two site-specific, high affinity photolabile analogs of SP (incorporating BPA in the third (BPA^3) or eighth (BPA^8) position) to label the SP receptor of $P388D_1$ cells and map the peptide-binding domains of the receptor for each ligand.

EXPERIMENTAL PROCEDURES

Preparation of Fluoren-9-ylmethoxycarbonyl-4-benzoylphenylalanine (Fmoc-BPA)—Racemic DL-BPA was synthesized and resolved into L- and D-BPA as described by Kauer *et al.* (1986). The resolved amino acid (or alternatively the racemic mixture) was treated directly with Fmoc-chloroformate (Aldrich) or Fmoc-hydroxysuccinimide (Sigma) to provide the protected amino acid (Fmoc-BPA) for solid-phase synthesis.

Peptide Synthesis—Peptide synthesis of the SP analogs, BPA^3-SP and BPA^8-SP, was performed by our departmental Biopolymers Facility or by Quality Control Biochemicals (Hopkinton, MA) using a standard Fmoc solid-phase synthetic strategy (Maggio *et al.*, 1992). The crude synthetic peptide was then purified by reverse-phase high performance liquid chromatography (HPLC) using a C_{18} column (Vydac 4.6 × 250 mm, 5 μm, 300 Å) on a Waters Liquid Chromatographic System equipped with a variable wavelength UV detector. The column was eluted with a linear water-acetonitrile gradient (26–56% acetonitrile, 1%/3 min; 1.0 ml/min) containing 10 mM trifluoroacetic acid. The racemic peptide had two major UV active (254 nm) components of equal intensity. The L-BPA peptide was identified by elution position; that is, L-BPA^8-SP (synthesized from Fmoc-L-BPA) showed only one UV active peak which was coincident with the earlier eluting HPLC peak of DL-BPA^8-SP (synthesized from Fmoc-DL-BPA). Thus, the earlier HPLC peak corresponds to L-BPA^8-SP and the later peak to D-BPA^8-SP. In addition, Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanineamide) formed an adduct (Marfey, 1984) with isolated L-BPA that precedes that formed from D-BPA. When the L-BPA^8-SP and D-BPA^8-SP fractions were tested for receptor binding and biological activity, the earlier eluting peptide, *i.e.* L-BPA^8-SP, was much more active than the later eluting D-BPA^8 analog. By analogy to the greater activity of L-Phe^8-SP relative to D-Phe^8-SP (Fournier *et al.*, 1982), this result further confirms that the first of the paired HPLC peaks is the L-BPA-isomer. The L-BPA diastereomer has also been shown to precede the D-BPA diastereomer of other peptides in reverse-phase HPLC elution (Shoelson *et al.*, 1993).

The isolated L-BPA^8-SP was analyzed for purity and correct structure by amino acid analysis, laser desorption mass spectroscopy, and sequence. The peptide was sequentially $Y_{0.2}$ $R_{0.9}$ $P_{0.7}$ $K_{1.0}$ $P_{0.7}$ $Q_{1.0}$ $Q_{1.0}$ $F_{1.2}$ (BPA) $G_{1.0}$ $L_{1.0}$ $M_{0.8}$ with m/z 1616.2 ($(M+H)^+$). Neither BPA or its phenylthiohydantoin derivative elute from the analyzer column under standard conditions (Kauer *et al.*, 1986). Nevertheless, the high UV extinction coefficient of BPA at 254 nm, $\epsilon = 21 \times 10^3$, verifies that BPA is incorporated (Kauer *et al.*, 1986), confirming the results of mass spectrometry. Tyrosine was partially destroyed under the hydrolysis conditions employed.

Preparation of Radioligands—The radioligand [^{125}I]BPA^8-SP ([^{125}I]iodotyrosyl^0-L-BPA^8-SP) was formed using general peptide iodination techniques previously described (Too and Maggio, 1991). Typically 10 nmol of dry peptide was dissolved in 50 μl of 0.5 M sodium phosphate buffer, pH 7.5, and vortexed with 1 mCi of Na^{125}I (10 μl, Amersham Corp.). Chloramine-T (10 μg in 10 μl of water) was added to activate iodine incorporation, and $Na_2S_2O_5$ (100 μg in 10 μl of water) was added after 1 min of vortexing to quench the reaction. The mixture was diluted and acidified with 0.6 ml of 60 mM trifluoroacetic acid, and 25 μl of 2% bovine serum albumin was added to limit nonspecific adsorption. To separate the peptide from the unincorporated ^{125}I, the mixture was then applied to an activated C_{18} Sep-Pak cartridge (Waters) and the iodide and peptide eluted with a series of 0.5-ml portions of 10 mM trifluoroacetic acid solutions of increasing alcohol (ethanol/methanol, 1:1) content, 10, 10, 20, 40, 60, 80, 90, 95, and 100%. Unincorporated iodide elutes immediately, while the peptide is retained until the alcohol concentration reaches about 60%. The peptide fractions (containing both oxidized and reduced methionine), eluting with 60–90% alcohol, were pooled and reduced in volume under a nitrogen stream. After the addition of 20% (v/v) β-mercaptoethanol, the sample was heated at 90 °C for 2 h to reduce methionine sulfoxide to its thioether. Further purification was achieved by reversed-phase HPLC on a Vydac C_{18} column as above. The eluate was collected in fractions during gradient elution and the fractions counted for radioactivity. The reduced (Met) and monoiodinated (Tyr) peptide eluted in a well-resolved peak (34.6% acetonitrile), predictably later than the original compound (33.2%) or the oxidized products, but prior to the diiodinated reduced peptide. The reduced monoiodinated tracer (specific activity −2000 C_i/mmol; $1C_i$ = 37 GBq) was protected from oxidation by 0.5% β-mercaptoethanol (v/v) added immediately after purification and stored at −20 °C until use. Radioiodinated BPA^3-SP was prepared similarly.

Cell Culture—The murine cell line $P388D_1$ (Dawe and Potter, 1957; Persico *et al.*, 1988) was a gift of Dr. J. Jackie (Harvard Medical School) and has been maintained in our laboratory in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS).

Calcium Measurements—$P388D_1$ cells were cultured on 12-mm diameter round glass coverslips that had been pretreated with laminin. These were used within 24–48 h after plating. Dye loading was achieved by exposing the cells to fura-2 acetoxymethyl ester at a concentration of 8 μM for 30 min at room temperature. The cells were then washed with 2% bovine serum albumin and kept on ice until used. Experiments were performed using a standard saline buffer with the following components: NaCl 120 mM, KCl 4.2 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.0 mM, Na_2HPO_4 1.0 mM, glucose 12 mM, and HEPES 10 mM, pH 7.40.

Fluorescence measurements were made using a Nikon microscope optically linked to a PTI Deltascan instrument (Photon Technologies) that produces dual excitation at 340 and 380 nm. Emitted light was collected after passing though a 510-nm band pass filter. A 40X Nikon fluor objective was used and the field was limited to about 15–20 cells for data collection.

Ligand Binding of $P388D_1$ Cells—$P388D_1$ cells (5 × 10^5 cells/well) were inoculated on FCS precoated 24-well plates and cultured overnight. The confluent cells (~1 × 10^6 cells/well) were washed twice (0.5 ml/well) with ice-cold buffer (Dulbecco's modified Eagle's medium + 20 mM HEPES, pH 7.2) and incubated with 0.5 ml of buffer on ice for at least 10 min. Then radioactive ligand, in the presence or absence of unlabeled displacers, was added to a final concentration of 150 pM (~5 × 10^5 cpm/ml) and incubated for 2 h. Nonspecific binding is defined as binding in the presence of 10 μM unlabeled SP. After incubation, the cells were washed twice with 0.5 ml/well phosphate-buffered saline (104 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2), then solubilized by 0.5 ml of lysis buffer (1% Nonidet P-40, 0.2% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0) for 20 min and transferred for gamma counting. Unlabeled SP and CP-96345 were stored as 10 mM stock solutions in dimethyl sulfoxide. Dimethyl sulfoxide at less than 3% (v/v) had no detectable effects on the binding assay.

Photoaffinity Labeling of $P388D_1$ Cells—Cells (5 × 10^6) were inoculated on FCS-pretreated dishes (60 mm) and grown for at least 12 h before labeling. The cultured cells (~1 × 10^7) were washed twice (5 ml) with ice-cold buffer (Dulbecco's modified Eagle's medium + 20 mM HEPES, pH 7.2) and incubated with 5 ml of buffer on ice for at least 10 min. The photolabile radioligand, in the presence or absence of un-

[2] H.-P. Too and J. E. Maggio, unpublished results.

labeled displacers, was added to a final concentration of 2 nM (~6 × 10^6 cpm/ml) and incubated 2 h. The dishes were then irradiated on ice for 15 min using a focused HBO 100-watt mercury short arc lamp through an optical filter to eliminate light below 310 nm. A second filter removed infrared wavelengths to minimize sample heating during photolysis.

After photolysis, the cells were washed twice (5 ml) with phosphate-buffered saline and transferred to a microcentrifuge tube to collect cell pellets by centrifugation at 16,000 × *g* for 10 min. The pelleted cells were resuspended in 0.3 ml of 5 mM Tris-HCl, pH 8.0, and hypotonically lysed for 30 min at room temperature. Then the samples were homogenized and centrifuged at 500 × *g* for 15 min to remove debris. The resulting supernatants were sedimented at 16,000 × *g* for 30 min and the membrane pellets stored at −20 °C until analysis. The presence or absence of a mixture of protease inhibitors (bacitracin, chymostatin, and leupeptin) did not affect the results of binding or photolysis experiments.

Partial Purification of the Labeled Complex—SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970) using 1.5-mm 8% gels. The labeled cell membranes were solubilized in 1 × SDS sample buffer (10% glycerol, 5% 2-mercaptoethanol, 3% SDS, 0.025% bromphenyl blue, 125 mM Tris-HCl, pH 6.8) for 30 min at room temperature. After electrophoresis, the gel was directly dried on a filter paper and exposed to x-ray film (Kodak XAR-5) with an intensifying screen (DuPont). The labeled bands were isolated from the preparative gel using a passive elution protocol similar to that described by Blanton and Cohen (1994). After autoradiography, radioactive bands of the labeled complex were excised from dried gels and rehydrated with extraction buffer (0.1% SDS, 100 mM NH_4HCO_3, pH 7.8). The gel slices were macerated and eluted for 1–4 days with extraction buffer. The eluted protein was filtered (Whatman No. 1) and concentrated using Centriprep-10 or Centricon-10 (Amicon). Finally, the labeled complex was precipitated by cold acetone (85–90%, v/v) overnight at −20 °C. The precipitate was dried and stored at −20 °C until use. For all electrophoretic gels, the ratio of bis-acrylamide to acrylamide was 3%. For gels above 12% acrylamide, 1% glycerol was added to the running buffer to prevent cracking of gels during drying.

Endoglycosidase F Digestion of the Partially Purified Complex—The acetone precipitate was resuspended in 10 mM EDTA, 0.1% SDS, 0.5% *N*-octylglucoside, 100 mM NH_4HCO_3, pH 7.8, and then digested with *Flavobacterium meningosepticum* endoglycosidase F (Endo F) (Boehringer Mannheim) for 2 days at room temperature.

Protease Digestion of the Partial Purified Complex—The acetone precipitate was resuspended in 0.1% SDS, 100 mM NH_4HCO_3, pH 7.8, and then digested with *Staphylococcus aureus* V8 protease (V8) (Boehringer Mannheim) for 2–4 days at room temperature or L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated bovine trypsin (Sigma) for 1–4 days at room temperature. Both BPA^3-SP and BPA^8-SP completely resist cleavage by these proteases under these conditions (not shown) as both ligands lack glutamic acids residues, and basic residues are protected by adjacent prolines (Fig. 1).

HPLC of Enzymatic Digests—The V8-digested samples were loaded into a Vydac C_8 column (2.1 × 150 mm, 5 μm, 300 Å) and eluted with increasing solvent B (0.09% trifluoroacetic acid in 60% acetonitrile, 40% 2-propanol) in solvent A (0.1% trifluoroacetic acid in water) at a flow rate of 0.25 ml/min. The elution of ^{125}I-labeled SPR fragments was monitored by gamma counting of the fractions. More than 90% of injected radioactivity was recovered in the eluate for all HPLC experiments.

5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) Modification—The HPLC fractions were dried and resuspended in 100 mM NH_4HCO_3, pH 7.8. Dithiothreitol was added to a final concentration of 25 mM and the mixture incubated for 30 min at 50 °C, then DTNB (final concentration 75 mM) was added and allowed to react with the peptide for at least 30 min. The mixture was then diluted with 20% solvent B and loaded onto reverse-phase-HPLC as described.

RESULTS

Specificity of BPA^3-SP and BPA^{8}SP—Both BPA^3-SP and BPA^8-SP (Fig. 1) are full and potent agonists relative to SP for the calcium responses of P388D$_1$ cells. This action is completely blocked by CP-96345, a specific nonpeptide antagonist of the SP receptor (Fig. 2). Both photoreactive ligands bound to SPR (Table I) with the same affinity (IC_{50} values ~3 nM) as SP and the binding of each was similarly inhibited by CP-96345 (IC_{50} values ~35 nM).

Photoaffinity Labeling of SP Receptor—After photoinsertion of radioiodinated BPA^3-SP or BPA^8-SP bound to P388D$_1$ cells, two radioactive bands were observed on SDS-PAGE. A major broad band of 75 kDa accounted for about 95% of the radioactivity, while a minor band of 205 kDa accounted for about 5% (Fig. 3). Labeling of both bands was completely inhibited by SP (Fig. 3) or CP-96345 (not shown). For BPA^8-SP, 46 ± 3% of bound ligand is recovered in the broad 75 kDa band; for BPA^3-SP, the major band represents about 6% of the total bound radioactivity. For both ligands, deglycosylation with Endo F shifted the broad major band to a sharp band of 42 kDa (Fig. 4), indistinguishable from the molecular mass of the murine SPR calculated from its cDNA sequence (Sundelin *et al.*, 1992). The major band labeled by both photoprobes was partially purified by preparative SDS-PAGE and used for further studies. Incubation of cells with the photoprobes in the dark resulted in no detectable incorporation into protein.

V8 Digestion of the Labeled SP Receptor—V8 protease (Glu-C) cleaves proteins specifically at the COOH-terminal side of glutamate residues under the conditions employed. V8 digestion of partially purified [^{125}I]BPA^8-SP-labeled SPR showed five detected bands (33, 25, 19, 9, and 3.2 kDa). The larger proteolytic fragments were converted into the smaller ones at higher concentration of V8 protease, with the smallest fragment, designated BPA^8-SPR-3.2k, being the limit digest (Fig. 5*A*). Double digestion of [^{125}I]BPA^8-SP-labeled SPR with Endo F and V8 protease revealed the same five-band pattern seen

Agonist	Residue Number											
	0	1	2	3	4	5	6	7	8	9	10	11
SP		Arg-	Pro-	Lys-	Pro-	Gln-	Gln-	Phe-	Phe-	Gly-	Leu-	Met- NH_2
BPA^3-SP	Tyr-	Arg-	Pro-	BPA-	Pro-	Gln-	Gln-	Phe-	Phe-	Gly-	Leu-	Met- NH_2
BPA^8-SP	Tyr-	Arg-	Pro-	Lys-	Pro-	Gln-	Gln-	Phe-	BPA-	Gly-	Leu-	Met- NH_2

FIG. 1. **Primary structures of SP, BPA^3-SP, and BPA^8-SP.** Addition of Tyr at the NH_2 terminus facilitates radioiodination. Lys^3 and Phe^8 are respectively replaced by BPA to give BPA^3-SP and BPA^8-SP.

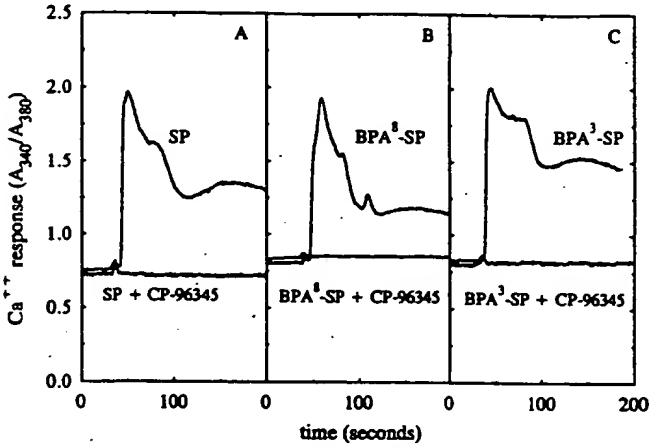

FIG. 2. **SP, BPA^3-SP, and BPA^8-SP induce calcium responses in P388D$_1$ cells.** $[Ca^{2+}]_{i\text{-in}}$ was measured with fura-2 using 12–18 cells in an optical field. Agonists (10 nM) were perfused over the cells for 30 s. CP-96345 (1 μM) was preincubated with the cells for 30 s before adding the mixture of agonist and CP-96345. Experiments were carried out at room temperature (22 °C).

TABLE I
IC_{50} values of substance P and CP-96345 for three ligands binding to P388D$_1$ cells

Ligands[a]	IC_{50} (nM)[b]	
	Substance P	CP-96345
[^{125}I]BHSP	2.30 ± 0.40	33.3 ± 11.5
[^{125}I]BPA^8-SP	2.75 ± 0.95	33.3 ± 11.5
[^{125}I]BPA^3-SP	3.00 ± 1.00	36.7 ± 5.70

[a] Final concentration 0.15 nM.
[b] Mean ± S.D. of at least three experiments.

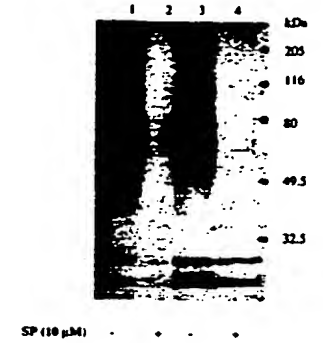

FIG. 3. **Autoradiography of P388D$_1$ cell membranes photoaffinity labeled with [^{125}I]BPA^3-SP and [^{125}I]BPA^8-SP following SDS-PAGE (8% gel).** Cell culture, photolysis, cell membrane preparation, and solubilization were as described under "Experimental Procedures." After electrophoresis, the gel was stained with 0.1% Coomassie Blue in MeOH/AcOH/H_2O (4:1:5) and destained in the same solvent. The same amount of protein was found in each lane (not shown). *Lanes 1* and *2*, [^{125}I]BPA^8-SP-labeled membranes; *lanes 3* and *4*, [^{125}I]BPA^3-SP-labeled membranes. The labeling was carried out in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of 10 μM unlabeled SP. Molecular weights are indicated at the *right*.

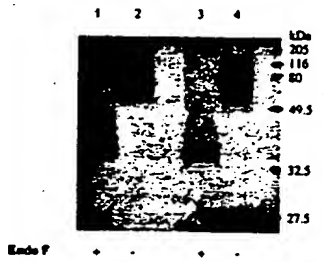

FIG. 4. **Autoradiography of Endo F deglycosylated photolabeled SP receptor following SDS-PAGE (12% gel).** Acetone-precipitated ^{125}I-labeled SPR (5000–8000 cpm) from preparative SDS-PAGE was dissolved in 0.1% SDS, 10 mM EDTA, 0.5% *N*-octylglycoside, 100 mM NH_4HCO_3, pH 7.8, and digested by Endo F (0.8 unit) for 2 days at room temperature. The gel was directly dried without fixation or staining prior to autoradiography. *Lanes 1* and *2*, [^{125}I]BPA^8-SP-labeled SPR; *lanes 3* and *4*, [^{125}I]BPA^3-SP-labeled SPR. The samples were treated with (*lanes 1* and *3*) or without (*lanes 2* and *4*) Endo F. Molecular weights indicated are at the *right*.

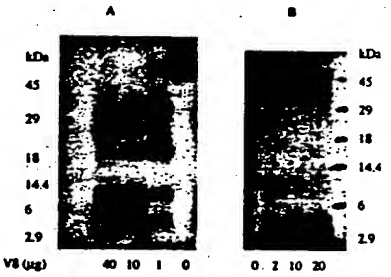

FIG. 5. **Autoradiography of V8 protease digest of photolabeled SP receptor following SDS-PAGE.** Acetone precipitated [^{125}I]-labeled SPR (5000 8000 cpm) from preparative SDS-PAGE was dissolved in 0.1% SDS, 100 mM NH_4HCO_3, pH 7.8, and digested by the indicated amount of V8 protease for 4 days at room temperature. The gel was directly dried for autoradiography. *A*, [^{125}I]BPA^8-SP-labeled SPR (17.5% gel); molecular weights are indicated at the *left*. *B*, [^{125}I]BPA^3-SP-labeled SPR (18% gel); molecular weights are indicated at the *right*.

with V8 digestion alone.

Peptide fragments from V8 digestion of [^{125}I]BPA^8-SP-labeled SPR were isolated by HPLC (Fig. 6*A*). One major peak (~33% solvent B), accounting for most of the eluted radioactivity, corresponded to BPA^8-SPR-3.2k (Fig. 6*B*). BPA^8-SPR-3.2k and BPA^8-SP tracer eluted in a similar position on reverse-

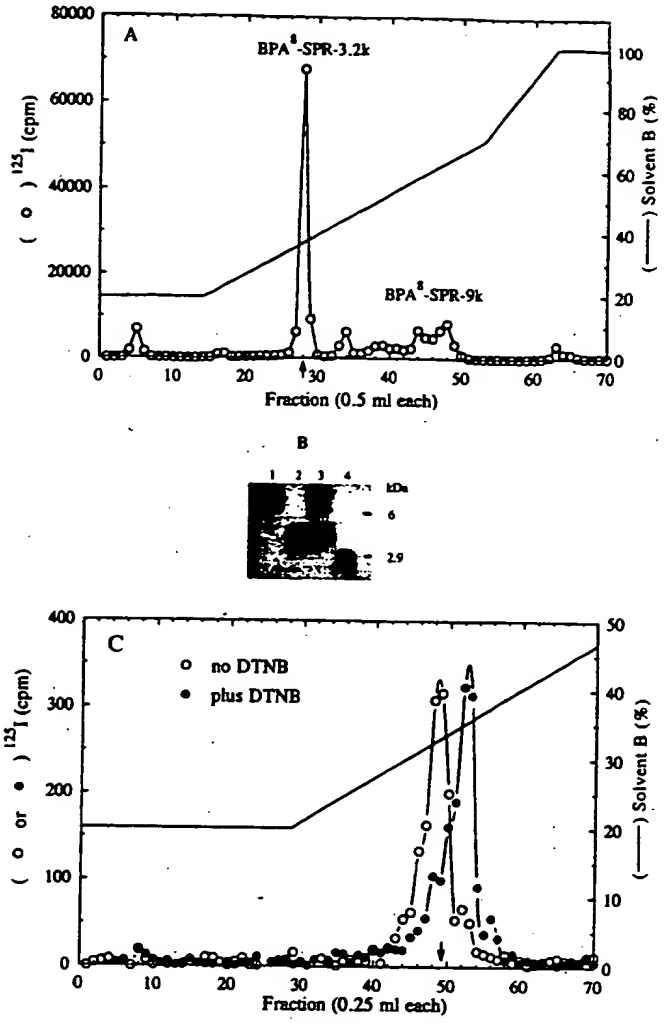

FIG. 6. **Analysis of the BPA^8-SPR-3.2k fragment.** [^{125}I]BPA^8-SP-labeled SPR was digested by V8 protease as described in Fig. 5. The digests were separated by reverse-phase HPLC as described under "Experimental Procedures" (microbore C_8 column, organic phase 60% acetonitrile, 40% isopropanol). The solvent gradient (flow rate 0.25 ml/min) was as follows: 20–70% solvent B in 62.5 min; 70–100% solvent B in 15 min. Fractions (0.5 ml each) were counted for ^{125}I (*A*). The digests and HPLC fractions were dried and analyzed by SDS-PAGE on an 18% gel (*B*). *Lane 1*, minor HPLC peak, fraction 44–48; *lane 2*, major HPLC peak, fractions 27–29; *lane 3*, V8 digest prior to HPLC fractionation; *lane 4*, [^{125}I]BPA^8-SP; molecular weights are indicated at the right. BPA^8-SPR-3.2k fractions were dried and treated with or without DTNB as described, then separated by HPLC using a solvent gradient of 20 50% solvent B in 37.5 min (*C*). *Arrow* indicates the elution position of [^{125}I]BPA^8-SP.

phase HPLC but were cleanly resolved by SDS-PAGE (Fig. 6*B*). A second peak of radioactivity eluting from the HPLC column, accounting for most of the recovered radioiodine not in BPA^8-SPR-3.2k, corresponded to BPA^8-SPR-9k. Further digestion of BPA^8-SPR-9k with V8 protease converted this fragment to BPA^8-SPR-3.2k (not shown).

BPA^8-SPR-3.2k, the limit digest, was reacted with DTNB, a specific sulfhydryl modification reagent which converts free peptidyl -SH groups to mixed disulfides of 2-nitro-5-thiobenzoic acid. Treatment of BPA^8-SPR-3.2k with DTNB shifted the HPLC elution position of the peptide to later elution by 3.2% solvent B (Fig. 6*C*). A parallel sample incubated identically but without DTNB showed no change in elution position.

V8 digests of SPR photolabeled with [^{125}I]BPA^3-SP displayed a different pattern of proteolytic fragments than digests of SPR photolabeled with [^{125}I]BPA^8-SP (Fig. 5). A major fragment of

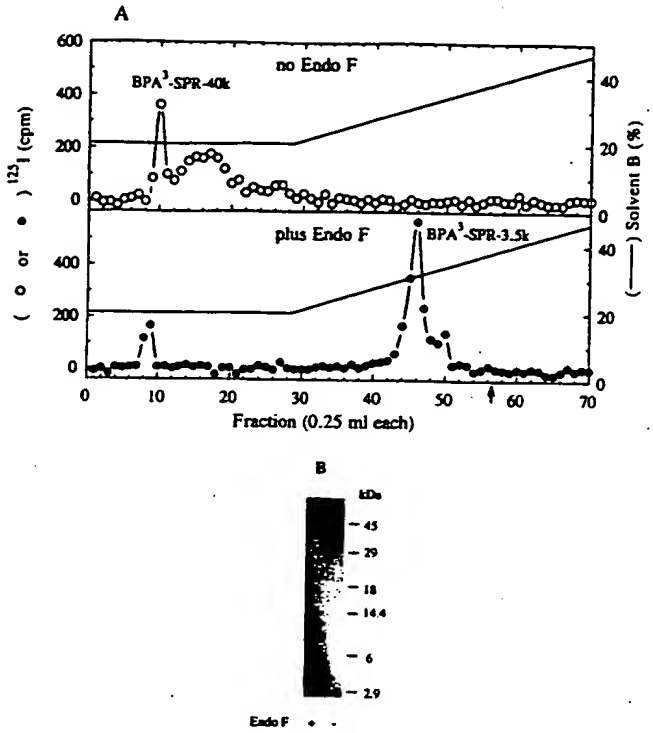

FIG. 7. **Analysis of the BPA³-SPR-40K fragment.** [¹²⁵I]BPA³-SP-labeled SPR was digested by V8 protease as described in Fig. 5. The digests were separated by HPLC as described in Fig. 6A. Fractions eluting at the void volume (unretained by the reverse-phase column) were dried and treated with or without Endo F. The samples then reanalyzed by HPLC (*A*) as described in Fig. 6C and by SDS-PAGE (*B*) on an 18% gel. *Lane 1*, HPLC fractions 43–48 after Endo F; *lane 2*, HPLC fractions 9–19 without Endo F; molecular weights are indicated at the *right*. *Arrow* (*A*) indicates the elution position of [¹²⁵I]BPA³-SP.

40 kDa (BPA³-SPR-40k) was detected under conditions which reduced SPR photolabeled with [¹²⁵I]BPA⁸-SP to peptides of less than 10 kDa. Very high concentrations of V8 produced, in addition to the major band at 40 kDa, minor bands at about 3 and 10 kDa. Endo F digestion of BPA³-SPR-40k produced a 3.5-kDa fragment, BPA³-SPR-3.5k. The same fragment was produced by double digestion with Endo F and V8 protease of SPR photolabeled with [¹²⁵I]BPA³-SP.

V8 protease-digested fragments of [¹²⁵I]BPA³-SP-labeled SPR were separated by HPLC. A major peak containing >70% of eluted radioactivity passed through without being retarded by the reverse-phase column. These fractions were dried, digested with Endo F, and reanalyzed by HPLC. The Endo F-treated sample (BPA³-SPR-3.5k) was retained by the HPLC column and eluted at 30% solvent B (Fig. 7). BPA³-SPR-3.5k and BPA³-SP were separable by HPLC. A control sample (identically treated in the absence of Endo F) still passed through the HPLC column without retention.

Trypsin Digestion of the Labeled SP Receptor—Double digestion of [¹²⁵I]BPA⁸-SP-labeled SPR with Endo F and trypsin revealed the same pattern of radioactive fragments as seen with trypsin digestion alone. However, double digestion (with Endo F and trypsin) of [¹²⁵I]BPA³-SP-labeled SPR produced a different pattern of fragments than that seen with trypsin treatment alone. As seen with Endo F and V8, digestion of the major high molecular mass (>45 kDa) tryptic fragment of [¹²⁵I]BPA³-SP-labeled SPR with Endo F converted it to a much smaller fragment.

DISCUSSION

Replacement of amino acid residues at the third (Lys^3) or eighth (Phe^8) positions of SP by BPA and addition of Tyr at the NH_2-terminal (Tyr^0) gave analogs (Fig. 1) which triggered calcium responses of $P388D_1$ cells (Fig. 2) with the same potency as the parent peptide. The calcium response was inhibited by CP-96345, a specific SPR antagonist. Binding of ¹²⁵I-labeled BPA⁸-SP and BPA³-SP to $P388D_1$ cells was blocked by cold SP and CP-96345 at nM concentrations (Table I). The two photolabile ligands thus are high affinity full agonists of the SPR of $P388D_1$ cells. Previous studies showed that Phe^8 of SP could be structurally modified without a marked decease in activity on affinity on several bioassays (Lee *et al.*, 1983; Maggio, 1988; Viger *et al.*, 1983). Boyd *et al.* (1991a, 1991b) have demonstrated that replacement of Phe^8 of SP by BPA is well tolerated in binding affinity and biological activity. The results of the present study illustrate that substitution of Lys^3 of SP with BPA also maintains biological activity and binding affinity at the SPR of murine $P388D_1$ cells. To facilitate radioiodination, we added a tyrosine at the NH_2 terminus of the peptide; the Tyr^0 peptides also retain full biological activity and binding affinity (Sachais *et al.*, 1993; Cascieri *et al.*, 1994).

Upon near UV irradiation (>310 nm), ¹²⁵I-labeled BPA⁸-SP and BPA³-SP are photoincorporated into a major broad radiolabeled band of ~75 kDa in $P388D_1$ cells (Fig. 3). The broad range of molecular mass reflects heterogeneous glycosylation, as Endo F treatment dramatically converted the broad 75 kDa band to a sharp one of ~42 kDa (Fig. 4). This size is consistent with the value deduced from the cDNA sequence for the mouse SPR (Sundelin *et al.*, 1992). The sensitivity of photolabeling with these ligands to SP and CP-96345 (Table I, Fig. 3) further indicated that BPA⁸-SP and BPA³-SP were cross-linked with the SPR.

This intact cell-photolabeling technique demonstrates that the SPR expressed in this natural (*i.e.* nontransfected) cell line is highly glycosylated. Reports of photolabeling of SPR prepared from various other sources suggests a heterogeneity of molecular size. Dam *et al.* (1987), using a photoreactive SP analog in which Phe^8 was replaced by *p*-azidophenylalanine, demonstrated specific photolabeling of a single polypeptide, ~46 kDa, in a rat brain membrane preparation. Boyd *et al.* (1994) reported that the molecular mass of SPR in rat tissues labeled with ¹²⁵I-3-(3-iodophenyl-4-hydroxylphenyl)propionyl-ε-Lys^3-BPA⁸-SP varied from 53 and 46 kDa for submaxillary or parotid gland to 72 kDa for large intestine and 90 kDa for striatum or olfactory bulb. Deglycosylation of each of these photolabeled receptors from different tissues yielded a discrete radiolabeled band of ~46 kDa, while in salivary gland an additional band at ~36 kDa was also observed.

Limited V8 digestion of [¹²⁵I]BPA⁸-SP-labeled SPR implies that the smallest labeled complex, BPA⁸-SPR-3.2k, represents the interaction site of the ligand and receptor, as all other fragments are converted to the 3.2-kDa fragment at high concentrations of protease. Since BPA⁸-SP has a molecular mass of ~1.7 kDa, a SPR V8 fragment peptide with molecular mass of ~1.5 kDa is involved in the BPA⁸-SPR-3.2k complex. DTNB reaction indicates that this peptide contains a cysteine residue. The V8 digestion map of the mouse SPR deduced from its cDNA sequence shows only four cysteine-containing peptides, with values of 10.7 kDa (SPR 79–172, 2 Cys), 1.3 kDa (SPR 173–183, 1 Cys), 3.8 kDa (SPR 194–227), 1 Cys), and 11.1 kDa (SPR 239–312, 6 Cys). The two larger fragments (M_r > 10 kDa) are excluded based on their molecular masses. Of the remaining two, the smaller (1.3 kDa) is clearly a much better candidate than the larger (3.8 kDa) for the ~1.5-kDa fragment deduced from the V8 digestion studies.

Consistent with the size prediction, HPLC elution behavior confirms that SPR 173–183 (1.3 kDa) is the photolabeled receptor fragment. SPR 173–183 is a part of the relatively polar second

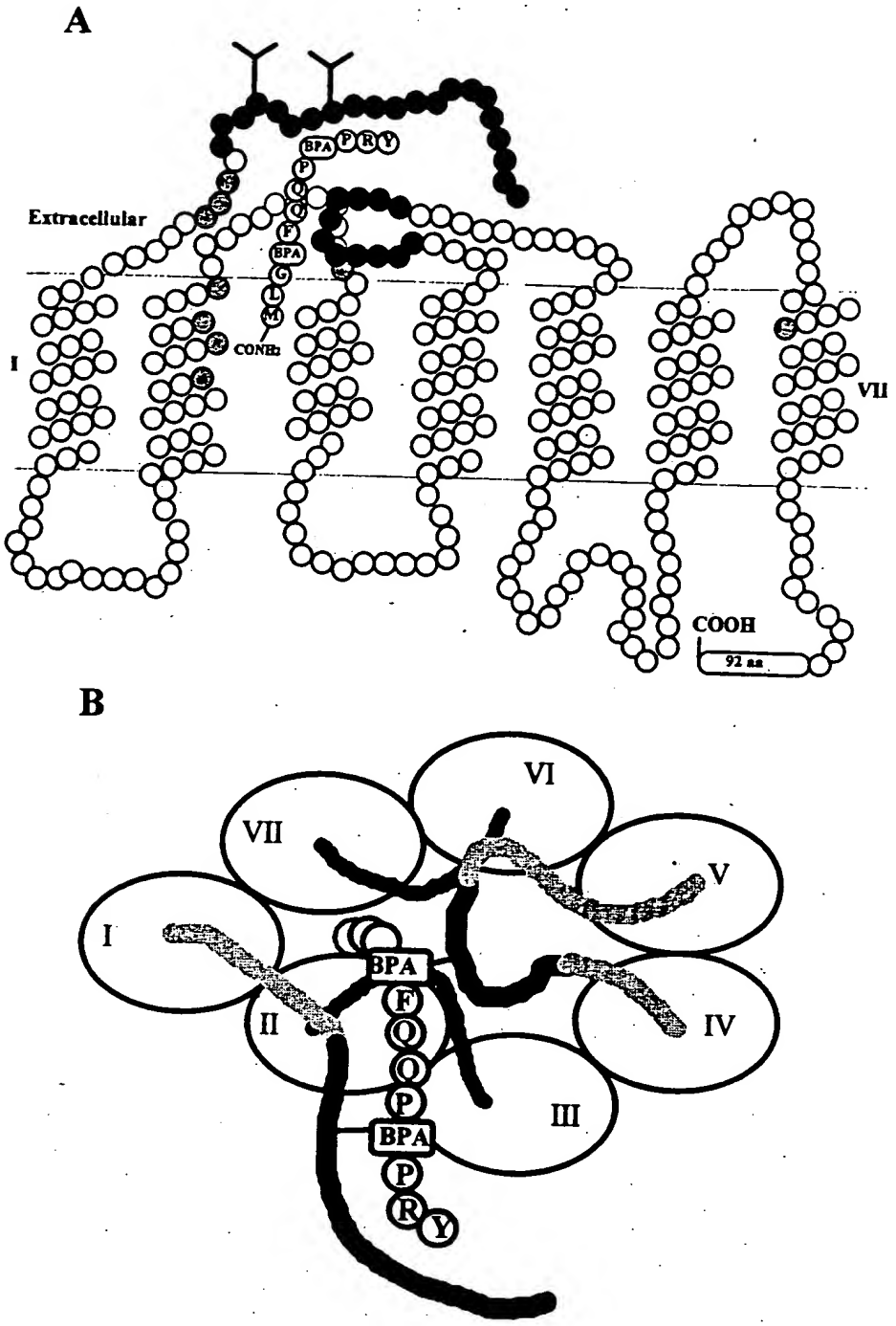

FIG. 8. **Schematic model of the peptide agonist binding site of the murine SP receptor.** *Black circles* represent the contact regions of SPR with SP analogs (BPA^3 and BPA^8). The third and the eighth positions of SP, respectively, interact with the NH_2-terminal extracellular tail (SPR 1–21, MDNVLPVDSDLFPNTSTNTSE) and second extracellular loop (SPR 173–183, TMPSRVVCMIE) of the SP receptor. *Shaded circles* indicate residues essential for high affinity binding of SP as identified by site-directed mutagenesis. *A*, view in the plane of the bilayer; *B*, view from the extracellular side, normal to the plane of the bilayer. See text for further explanation.

extracellular domain of the SPR (between transmembrane domain (TM) IV and TMV), with a hydrophobic index (Engelman *et al.*, 1986) of −1.8. The addition of such a fragment to BPA^8-SP (hydrophobic index −15.1) would have little effect on HPLC elution position, as is observed experimentally (Fig. 6). Both the tracer alone, and its photoadduct with the receptor fragment, elute as sharp peaks at the same (~33% solvent B) position in the solvent gradient. In sharp contrast, SPR 194–227 (~3.8 kDa) is comprised mainly of the very hydrophobic TMV region of the receptor and has a hydrophobic index of 35. Hydrophobic peptides elute from reverse-phase HPLC columns as relatively broad peaks at very high solvent concentrations. For example, a photolabeled transmembrane domain (α-M4, residues 401–428, hydrophobic index = 36.9) from the *Torpedo* nicotinic acetylcholine receptor, with very similar molecular weight and amino acid composition to SPR 194–227, elutes in a very broad peak at about 74% solvent B in the same HPLC system (Blanton and Cohen, 1994). Such behavior is inconsistent with the reverse-phase-HPLC elution of BPA^8-SPR-3.2k (Fig. 6). Thus, molecular weight, HPLC elution behavior, and presence of cysteine, taken together, establish that the SPR region photolabeled by BPA^8-SP is SPR 173–183 of the second extracellular loop, whose primary sequence is TMPSRVVCMIE. Using a different SP tracer containing BPA at position 8, Boyd *et al.* (1993) also found labeling of the second extracellular loop of rat SPR in transfected hamster cells, a finding consistent with the present results. Because the radiolabel in these probes is located at a site distinct from the photoreactive amino acid, radiochemical sequencing cannot be used to define the specific amino acid of the SPR labeled by BPA.

Limited V8 digestion of [^{125}I]BPA^3-SP-labeled SPR suggested that the glycosylated peptide complex, BPA^3-SPR-40k, represents the interaction site of the ligand and receptor be-

cause Endo F plus V8 mixed digestion shifted the 40-kDa complex to a much smaller fragment, BPA^3-SPR-3.5k. This is further confirmed by HPLC analysis. BPA^3-SPR-40k passed though the reverse-phase column in the void volume, behavior common to very polar biopolymers such as carbohydrates. Deglycosylation of BPA^3-SPR-40k converted the complex to a smaller peptide (~3.5 kDa) which was retained by the reverse-phase column and eluted by the solvent gradient at about 30% solvent B. There are two potential sites (N-*X*-S/T) for *N*-linked glycosylation in the SP receptor. Both are located in the NH_2-terminal extracellular tail of the receptor, based both on primary sequence (Sundelin *et al.*, 1992) and experimental results (Boyd *et al.*, 1991b, 1994). The V8 digestion map of the murine SPR indicated that NH_2-terminal peptide (SPR 1–21, ~2.3 kDa) contains two *N*-linked glycosylation sites, while all other fragments have none. The hydrophobicity index of this (deglycosylated) peptide is −27.3, which predicts that the deglycosylated receptor fragment cross-linked with BPA^3-SP would elute earlier than the free photoprobe. Consistent with this prediction, the complex does elute earlier in the solvent gradient than [^{125}I]BPA^3-SP. Thus, molecular size of the complex, the presence of carbohydrate, and HPLC elution behavior, taken together, establish that the NH_2-terminal extracellular tail of the receptor (SPR 1–21, whose primary sequence is MDNVLPVDSDLFPNTSTNTSE) is the insertion site of [^{125}I]BPA^3-SP.

Photoaffinity labeling identifies receptor domains in close proximity to the bound photoligand. Another approach to receptor-ligand interactions, site-directed mutagenesis, identifies domains necessary for function, but which are not necessarily proximal to the site of that function. Chimeras of the substance P receptor with other tachykinin receptors (*e.g.* substance K receptor) demonstrated the agonist ligand specificity of the tachykinin receptors is mainly determined by the region around TMII TMIV and also partly by the extracellular NH_2-terminal domain of the receptors (Yoshifumi *et al.*, 1992). Fong *et al.* (1992b) found that extracellular domains of SPR (also known as NK-1R), including a segment of NH_2-terminal tail and the first extracellular loop were essential for high affinity binding of agonist peptides. Furthermore, they identified several residues in the NH_2-terminal domain (Asn^{23}, Gln^{24}, and Phe^{25}), first extracellular (also known as E2) loop (Asn^{96}, His^{108}), and part of second extracellular (also known as E3) loop (Ser^{176}-Glu^{83}) which are required for high affinity binding of peptides (Fong *et al.*, 1992a). Other mutagenesis studies demonstrated that residues in TMII (Asn^{85}, Asn^{89}, Tyr^{92}) and TMVII (Tyr^{287}) are also required for high affinity binding of peptide agonists (Huang *et al.*, 1994b). Analysis of SP analogs further suggested the COOH-terminal carboxyamide of SP may interact with Asn^{85} in the second transmembrane domain (Huang *et al.*, 1994b). Taken together, these data demonstrate that both the extracellular and transmembrane domains of SPR are important for the peptide binding. The present studies identify the interaction sites of the third (BPA^3) and eighth (BPA^8) positions of SP as (*i.e.* BPA^3 and BPA^8 contact and photolabel) the NH_2-terminal extracellular tail (SPR 1–21) and the second extracellular loop (SPR 173–183) of the receptor, respectively. The results of the present photolabeling experiments and those of previous mutagenesis experiments are distinct, in that the different regions of the SPR are identified, but not inconsistent. The photolabeling results do not match the predictions of a graphics-computer-generated model (Trumpp-Kallmeyer *et al.*, 1994) of SP bound to its receptor.

Combining the present results with those of previous mutagenesis studies, a model of the agonist peptide-binding site of the SPR can be constructed (Fig. 8). In this model, the COOH-terminal hydrophobic sequence -GLM-NH_2 of SP inserts into a hydrophobic ligand binding pocket between the transmembrane domains and between the extracellular surface and center of the bilayer. This binding pocket is formed by TMII and TMVII with contributions from other transmembrane domains. The carboxyamide penetrates to the level of and interacts with Asn^{85} (Huang *et al.*, 1994b). Other than this COOH-terminal tail, the remainder of the SP molecule interacts with amino acids on the extracellular face on the receptor. Specifically, position 8 of SP interacts with the second extracellular loop (SPR 173–183) and position 3 of SP with the NH_2-terminal extracellular tail (SPR 1–21). These regions of the SPR are highly conserved across species; 10 of 11 amino acids of SPR 173–183 and 18 of 21 amino acids of SPR 1–21 are invariant across the four mammalian species whose SPR cDNA sequences have been reported (Gerard *et al.*, 1993). The binding site for specific, high affinity nonpeptide antagonists of the SPR is at a distinct location (Cascieri *et al.*, 1994; Fong *et al.*, 1992a, 1992b, 1993, 1994a, 1994b; Gether *et al.*, 1993a, 1993b, 1993c, 1994; Huang *et al.*, 1994a; Jensen *et al.*, 1994; Sachais *et al.*, 1993; Yokota *et al.*, 1992; Zoffmann *et al.*, 1993).

Studies of other G-protein-coupled receptors have demonstrated that those which bind larger (>10 kDa) agonists have agonist-binding sites within their NH_2-terminal extracellular domains. In contrast, receptors of this superfamily which bind smaller (<0.5 kDa) nonpeptide agonists have agonist-binding sites deep within the bilayer between the transmembrane domains (Bockaert, 1991; Dohlman *et al.*, 1991). The smallest neuropeptide, thyrotropin-releasing hormone (360 Da) apparently also binds within this same region (Perlman *et al.*, 1994). Recently Gerszten *et al.* (1994) found that the specificity of thombin receptors for peptide agonists was determined by the extracellular face of the receptor. Substance P, a peptide agonist of intermediate size, apparently interacts with both the extracellular region and transmembrane region of its receptor. Thus, the regions of interaction between SP and its receptor include elements of both the large and small agonist-receptor systems. Other bioactive peptides among the dozens in this intermediate size range may similarly interact with both the extracellular and transmembrane domains of their own G-protein-coupled receptors.

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Miklos Bodanszky

Peptide Chemistry

A Practical Textbook

Second, Revised Edition

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To the memory of
my brother Dr. S. Bodanszky

should be applied in an excess that provides for a concentration of at least 10^{-1} M throughout the coupling reaction. This measure (the "principle of excess") counteracts the decrease in rate which necessarily occurs in bimolecular reactions as the concentration of the reactants decreases. Therefore the extent of unimolecular side reactions, in which the rate is independent of concentration, can be markedly reduced. An excess of acylating agent also helps to achieve complete acylation of the amine-component and prevents thereby the formation of "deletion sequences", peptides from which one amino acid residue is missing.

A further concentration related problem has to be mentioned here. Following deprotection by acidolysis the regenerated amine is isolated as a salt of the acid used for cleavage. In the subsequent acylation step, however, the free amine is needed. Deprotonation with the help of ion exchangers can be applied or, in solid phase peptide synthesis (Chapter X) treatment with a tertiary amine and removal of the trialkylammonium salts by washing. In syntheses carried out in solution the general practice is to "liberate" the amine-component from its salt by adding an equimolar amount of a tertiary amine (triethylamine, diisopropylethylamine, N-methylmorpholine or N-ethylpiperidine) to the reaction mixture prior to coupling. It is obvious, however, that often only an equilibrium

$$R-NH_3^+ + R'_3N \rightleftharpoons R-NH_2 + R'_3NH^+$$

can be established. While it is true that during acylation of the amino group this equilibrium is gradually shifted to the right, at any given time the concentration of the amine-component is lower than it would be if applied in completely deprotonated form. A notable exception is created by the insolubility of tertiary ammonium salts in certain solvents. For instance triethylamine hydrochloride, being practically insoluble in ethyl acetate, separates from the reaction mixture (if ethyl acetate is used as solvent for coupling) and shifts the equilibrium in the desired direction. The presence of tertiary amines is usually unfavorable during coupling: they can initiate side reactions through proton abstraction. These reactions can be suppressed by the addition of certain weak acids, for instance 2,4-dinitrophenol or pentachlorophenol, which show a distinct affinity for tertiary amines. They do not protonate the amine-component firmly enough to prevent its acylation. Moreover, the application of tertiary amines can be avoided by selecting highly acid sensitive amine-blocking groups and removing them with suitable weak acids, such as 1-hydroxybenzotriazole or tetrazole. The resulting salts are readily acylated, even with moderately reactive esters, without the addition of a tertiary base.

Additional Source

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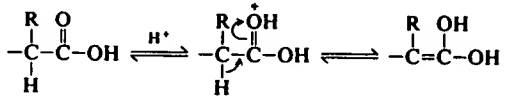
VIII. Racemization

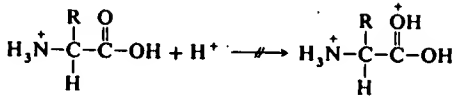
With the exception of glycine, in all amino acids that are constituents of proteins, the α-carbon atom is chiral. In threonine and isoleucine a chiral center is present in the side chain as well. In order to secure the target peptide in homogeneous form it is absolutely essential to start from enantiomerically pure amino acids and to insist on conservation of chiral homogeneity throughout the various operations of synthesis. Otherwise, instead of a single product, a mixture of stereoisomers will be obtained. Their number in a peptide with n chiral centers is 2^n. Accordingly, if racemization is not prevented, even in the synthesis of a moderately large peptide a complex mixture will be produced and separation of the desired material from a multitude of similar compounds might turn out to be an at least arduous and sometimes overwhelming task. Therefore, the importance of racemization studies and of the measures that must be taken for the prevention of any loss in chiral purity can not be overemphasized. In fact, "strategies" of peptide synthesis, that is general planning of schemes for syntheses (Chapter IX) are dictated primarily by considerations concerning conservation of chiral homogeneity.

A. Mechanism of Racemization

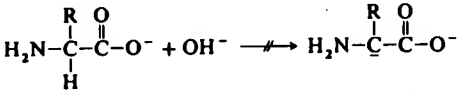
With respect to chiral stability amino acids are fairly insensitive to acids and bases. Racemization via enolization of carboxylic acids in acidic solutions involves protonation of the carbonyl oxygen



yet, the presence of a nearby positively charged nitrogen atom hinders the formation of the second cation:

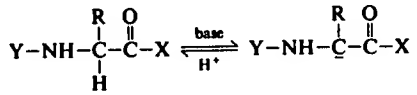


The same can be said about base-catalyzed racemization of amino acids. A negative charge on the carboxylate hinders further proton abstraction from the α-carbon of amino acids; dianions

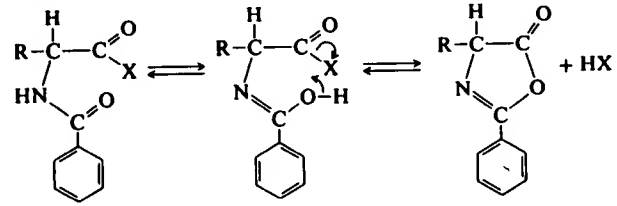


are not stable in protic solvents. Therefore, operations involving the amino acids themselves, for instance introduction of the benzyloxycarbonyl group, are carried out in distinctly alkaline solution. In fact, excess alkali prevents the formation of reactive derivatives, such as mixed anhydrides, which might be prone to racemization. In the absence of a free carboxyl group, as in alkyl esters of peptides, base catalyzed racemization does indeed occur during saponification with alkali.

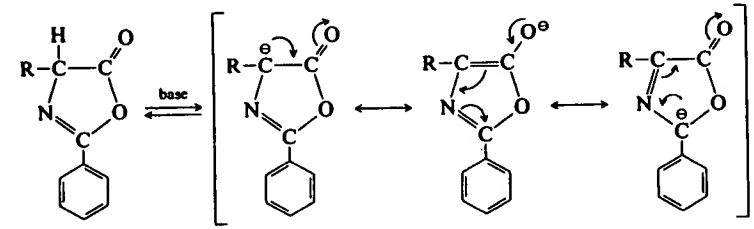
As indicated in the last paragraph, the *activated* carboxyl group poses the main problem in the preparation of optically homogeneous peptides. The electron-withdrawing effect of the activating group (x) extends to the α-carbon atom, the chiral center, and facilitates the abstraction of the hydrogen atom in the form of a proton



This kind of simple proton abstraction is, however, not the sole, and not even the most common, mechanism of racemization. The most frequently invoked pathway involves cyclic intermediates, 4,5-dihydro-oxazole-5-ones or *azlactones:*

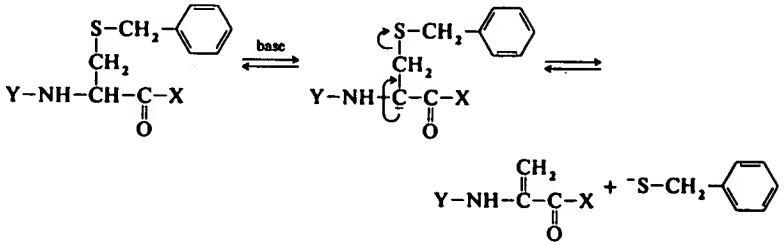


Proton abstraction from the chiral center yields a resonance stabilized anion

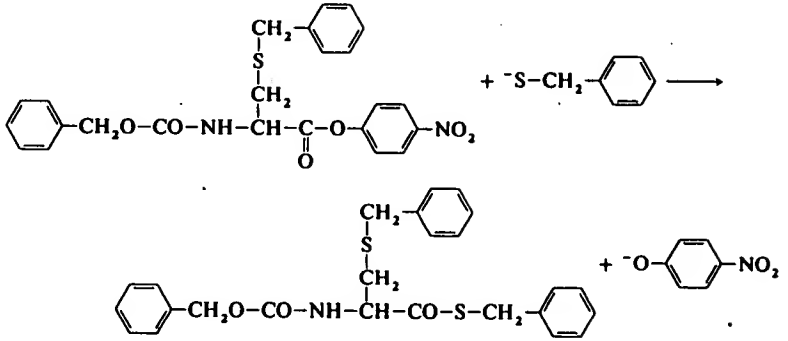


that was first postulated and subsequently proven by ir spectra. Azlactone formation is quite pronounced in benzoylamino acids, less prevalent in acetylamino acids and was for a long time thought to be absent in amino acids acylated by benzyloxycarbonyl or other urethane type blocking groups. The absence of racemization on activation of the latter was attributed to lack of azlactone formation, but in recent years azlactones were obtained from benzyloxycarbonyl-, tert.butyloxycarbonyl etc. amino acids as well. Furthermore, some optically pure azlactones were also prepared. Thus, azlactone formation itself is not a sufficient explanation of racemization; the stability of the cyclic intermediate toward bases must also be taken into consideration.

Activated derivatives of S-alkyl-cysteine suffer base catalyzed racemization even when their amino group is blocked by the benzyloxycarbonyl or other urethane-type protecting group. A simple, but not uncontested, explanation is reversible β-elimination



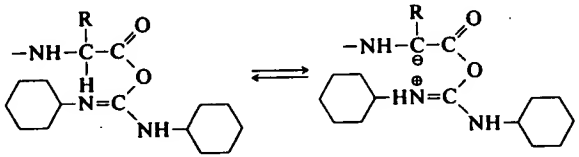
supported by the isolation of the thiobenzyl ester of N-benzyloxycarbonyl-S-benzyl-DL-cysteine. This indicates that benzylmercaptan, one of the products of β-elimination was present in the reaction mixture



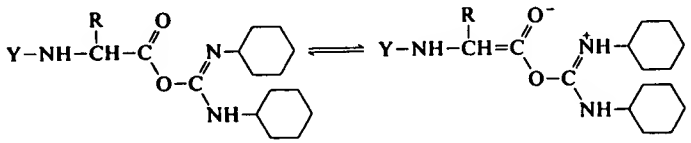
Experiments with S^{35} labeled benzylmercaptane, however, showed no incorporation of radioactivity. Also, racemization appears to be faster than deuterium exchange at the α-carbon atom. Thus racemization via β-elimination might occur only at elevated temperature, while other mechanism(s) could be opera-

tive under the conditions usually maintained during coupling. Among the various hypotheses that were put forward the partial acceptance of the negative charge of the carbanion intermediate by the d-orbitals of the sulfur atom is somewhat contradicted by the racemization of O-benzyl-serine derivatives.

The mechanisms described in the preceding paragraphs are the ones generally proposed for the explanation of racemization, but it is far from certain that other pathways are not involved. For instance it seems to be possible that the repeatedly observed loss of chiral integrity of the activated residue in coupling of peptides with the aid of dicyclohexylcarbodiimide is due to *intramolecular* proton abstraction by the basic center in the reactive O-acylisourea intermediate



or to enolization induced by the same strongly basic isouronium group:



Thus the acidic character of additives such as 1-hydroxybenzotriazole contributes to their ability to prevent racemization in coupling with carbodiimides.

B. Detection of Racemization

Loss of chiral homogeneity is an always present risk in peptide synthesis and there is an obvious need for methods that can reveal the presence of undesired diastereoisomers in the intermediates and particularly in the final product of the chain-building procedure. With carefully developed chromatographic systems it is often possible to separate fairly long peptide chains which are different from each other only with respect to the configuration of a single amino acid residue. There are however several methods available for this kind of scrutiny that can be applied without a special study of the particular product in question. Such general methods require hydrolysis of the peptide either with *acid* or with the aid of *proteolytic enzymes*. The specificity of these enzymes is the major advantage of the enzymatic approach: no hydrolysis occurs between a D-residue and the next amino acid in the sequence. Therefore complete hydrolysis will take place only in peptides that contain no D residues. The rate of peptide

bond fission, however, is a function of the amino acid cleaved from the N-terminus. Very slow rates can be achieved in the hydrolysis of glycyl and prolyl peptides with leucineaminopeptidase (a misnomer, since it is not specific for leucine), fewer difficulties are encountered with aminopeptidase M. Fast and complete hydrolysis of proline containing peptides requires the use of prolidases. A mixture of two enzymes, e.g. aminopeptidase M and prolidase can be quite efficient. Similar results are obtained with carboxypeptidases that provide stepwise removal of single amino acids starting with the C-terminal residue. Carboxypeptidase A has reduced catalytic effect when basic amino acids occupy the terminal position while carboxypeptidase B is most efficient just in this case. The yeast enzyme, carboxypeptidase Y is a more general catalyst.

A considerable number of biologically active peptides end with carboxamide rather than with a free carboxyl group. These peptide amides are, of course, no substrates for carboxypeptidases. An analogous problem exists in peptides which carry an acyl group such as the acetyl group at their N-terminus and, accordingly, can not be degraded with aminopeptidases. If both exopeptidase enzyme types fail, one can resort to a preliminary fragmentation of the chain with endopeptidases, such as trypsin. The latter is very specific for basic amino acids and catalyzes hydrolytic cleavage of the bond between arginine and the next residue and of the bond that follows lysine. The tryptic fragments then are suitable for further enzymatic degradation with exopeptidases, particularly with carboxypeptidase B. Chymotrypsin is similarly useful, but its specificity is somewhat less pronounced: in addition to the bond which follows an aromatic amino acid, the bond after a leucine residue is also cleaved, albeit at a slow rate. In hydrolyzates obtained with proteolytic enzymes only amino acids should be present; uncleaved peptides reveal the presence of a D-residue.

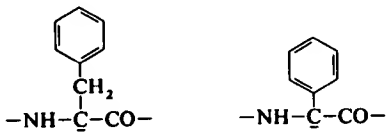
Acid catalyzed hydrolysis followed by the identification of D-amino acids in the hydrolysate is equally useful. To make this possible the amino acids in the mixture are acylated with an enantiomerically pure amino acid, for instance with the N-carboxyanhydride of L-leucine. In the resulting mixture of dipeptides any racemized residue is revealed by the formation of *two* dipeptides that are diastereoisomers of each other, for instance L-leucyl-L-phenylalanine and L-leucyl-D-phenylalanine. Since these are compounds with different physical properties they are separable and appear as a doublet on recordings of an amino acid analyzer. In recent years the conversion to diastereoisomers became unnecessary because the availability of chiral supports now permits separation of enantiomers by high pressure liquid chromatography (HPLC) and also by thin layer chromatography on plates covered with a chiral layer.

C. Racemization Studies in Model Systems

Racemization during the synthesis of peptides is a complex problem. The diversity of possible courses followed in the process is compounded by the

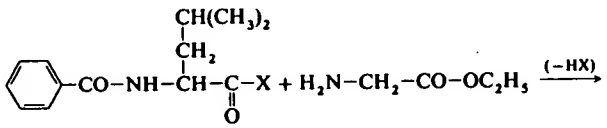
individuality of the amino acids. This was already shown in the example of S-alkyl-cysteine residues, which lose chiral purity by a special mechanism even if protected by a urethane-type protecting group that prevents racemization in other acylamino acids. The opposite end of the scale is represented by proline, which, at least under the commonly applied conditions of peptide synthesis, resists racemization. This was conventionally explained by the circumstance that proline is a secondary amine and, therefore, in its N-acyl derivatives lacks the hydrogen atom which participates in the formation of azlactones (cf. section A in this chapter). The experience, however, gained with readily racemized N-acyl derivatives of N-methylamino acids contradicts this assumption. It appears more likely that the rigidity of the cyclic side chain of proline excludes certain transition states that are integral parts of the racemization process.

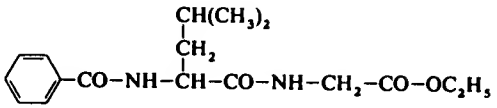
Various side chains affect the extent of racemization in different ways. Thus, the benzyl side chain in phenylalanine contributes to the stabilization of a carbanion and can thereby facilitate proton abstraction from the α-carbon atom. This effect is much more pronounced in phenylglycine (which is not a protein constituent but occurs in microbial peptides) because its chiral carbon atom is benzylic:



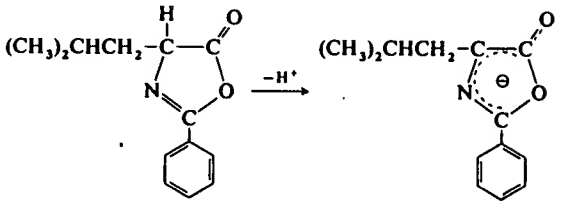
The aliphatic side chains in alanine and leucine have no major influence but branching at the β-carbon atom in valine and isoleucine can enhance racemization because the combination of electron release and steric hindrance results in reduced coupling rates. The ensuing increase in the life-time of the reactive intermediate provides an extended opportunity for proton abstraction by base. It is obvious from these examples that the effect of individual side chains, the influence of various methods of coupling and the conditions of the peptide bond forming reaction (solvents, concentration, temperature, additives) must be studied in well designed experiments. Several model systems have been proposed for this purpose.

The first model (Williams and Young 1963) was based on coupling of benzoyl-L-leucine to glycine ethyl ester. The specific rotation of the crude product was used





to establish enantiomeric purity. This simple system soon became popular and provided valuable information. Some shortcomings of the method must also be taken into consideration. The benzoyl group is not the best representative of blocking groups or of the part of the peptide chain that acylates the activated residue: it is more conducive to azlactone formation and might contribute to the stability of the anion generated from the azlactone by proton abstraction:

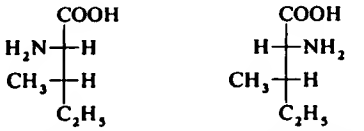


Therefore the Young-test might lead to somewhat exaggerated estimates of racemization. This distortion is counterbalanced by the relative resistance of leucine to racemization, but an additional problem is created by the necessity of isolating the crude benzoyl-leucyl-glycine ethyl ester in excellent yield. If less than near quantitative yield is achieved in coupling or in recovery of the product, there remains the possibility that the unaccounted portion contains a not insignificant amount of the D-isomer.

A frequently used early model (Anderson and Callahan 1958) is based on the coupling of benzyloxycarbonyl-glycyl-L-phenylalanine to glycine ethyl ester. Since the phenylalanine residue is acylated by glycine and not by the benzyloxycarbonyl group, it is not protected against racemization. Accordingly, reactions which cause loss of chiral purity produce in addition to Z-Gly-L-Phe-Gly-OEt also its enantiomer Z-Gly-D-Phe-Gly-OEt. The extent of racemization is easily established from the amount of the racemate because it separates from dilute ethanol. However, the results of this test are reliable only if the peptide bond forming reaction proceeds with excellent yield. The presence of byproducts can grossly interfere with crystallization and no racemate might separate although the D-isomer has been produced in considerable amount. In general: it is risky to rely on negative evidence, the *lack* of separation of the racemate.

Several later model systems were designed with the thought of separating products that are not enantiomers but diastereoisomers of each other. For instance in the coupling of acetyl-L-isoleucine to glycine ethyl ester racemization will yield acetyl-D-alloisoleucyl-glycine ethyl ester, because inversion at the

α-carbon atom leads to a D-amino acid while chirality at the second chiral center, the β-carbon atom is unaffected



L-isoleucine D-allo-isoleucine

(Fischer projections)

and hence, an alloisoleucine derivative is obtained. Complete hydrolysis (e.g. with 6 N HCl at 110° for 16 hrs) cleaves the amide and ester bonds and the hydrolysate can be applied to the column of an amino acid analyzer. In the well established Stein-Moore, method of amino acid analysis, isoleucine and alloisoleucine appear as well separated peaks and their ratio provides the information sought about the extent of racemization. The method does not require separation of the two peptides and therefore the results are not modified by imperfections in the operations of recovery. In more general versions of the same idea, diastereomeric tripeptides are produced, deblocked and compared with the help of the amino acid analyzer as such, that is without hydrolysis. For instance benzyloxycarbonyl-glycyl-L-alanine is coupled to L-leucine benzyl ester and after hydrogenation the mixture containing glycyl-L-alanyl-L-leucine and glycyl-D-alanyl-L-leucine is applied to the column of the instrument. By replacing L-alanine with other L-amino acids important information can be gained about the sensitivity of various amino acids to a certain coupling method or the conditions of coupling.

Volatile peptide derivatives, for instance trifluoroacetyl-L-valyl-L-valine methyl ester or benzyloxycarbonyl-L-leucyl-L-phenylalanyl-L-valine tert.butyl ester can be separated from their diastereoisomers that contain a D-residue by vapor phase chromatography. Also, through the examination of nmr spectra of relatively simple peptides the extent of racemization that occured during their preparation can be determined without separation of the diastereoisomers, because the difference in the chemical shifts of some selected resonances is sufficient for integration. Thus the areas under the well separated peaks of the alanine methyl protons in acetyl-L-phenylalanyl-L-alanine methyl ester and in acetyl-D-phenylalanyl-L-alanine methyl ester can be integrated and the values used to determine the extent of racemization of the phenylalanine residue during coupling.

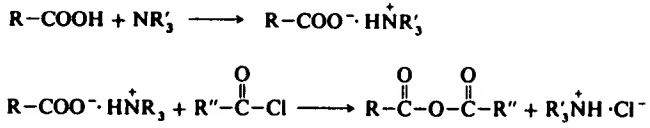
These are only selected examples of the numerous model systems proposed for the study of racemization, yet, even in such a brief treatment an approach based on enantio-selective enzymes should be mentioned. Coupling of benzyloxycarbonyl-L-alanyl-D-alanine to L-alanyl-L-alanine benzyl ester yields a blocked intermediate from which on catalytic hydrogenation the free L-Ala-D-Ala-L-Ala-L-Ala is obtained. This compound is completely resistant to hydrolysis catalyzed by aminopeptidases, because the first bond to be cleaved links the N-terminal residue to a D-amino acid. If however, racemization took place during coupling, this changed the activated residue, D-alanine to L-alanine and after deblocking the tetrapeptide L-Ala-L-Ala-L-Ala-L-Ala is obtained. The latter is completely digestible with aminopeptidases. The liberated alanine is determined and it is a rather exact measure of racemization because for each residue inverted four molecules of alanine are found in the analysis.

At this point a comment has to be added concerning the degree of racemization established with the help of model systems. Usually the amount of the undesired diastereoisomer is considered to represent the extent of racemization. While this might be acceptable for the purpose of comparisons, one has to keep in mind that from the achiral intermediate of the process the two isomers formed in equal amounts. Thus the number of molecules involved is twice the number of the undesired diastereoisomers formed.

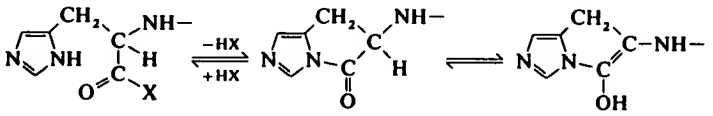
D. Prevention of Racemization

Since racemization during coupling is a base catalyzed process it is reasonable to assume that the nature of the base is not without influence on its outcome. Steric hindrance in some tertiary amines can weaken their ability to approach the chiral center in reactive intermediates. Diisopropylethylamine caused less racemization than triethylamine in the coupling of S-benzyl-L-cysteine derivatives, but it was without significant beneficial effect in reactions involving other amino acids. Perhaps in azlactones, the commonly implicated intermediates of racemization, the chiral carbon atom is well exposed and therefore the bulky groups in the tertiary amine can not interfere with proton abstraction. Also, some differences were found in couplings via mixed anhydrides between the previously preferred base triethylamine and tertiary amines such as N-ethylpiperidine or N-ethylmorpholine, the latter being less conducive to racemization. The principal lesson to be learned is, however, to omit, when possible tertiary amines from the coupling mixture. The often applied approach, addition of a tertiary amine to a salt of the amine component, is certainly inferior to the use of the amine component as such, that is the free amine. Several studies demonstrated that very little if any racemization takes place if this simple principle is followed.

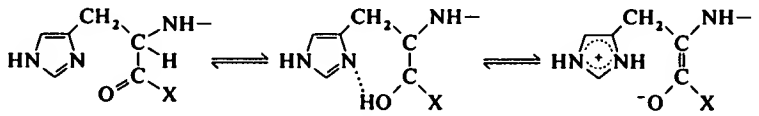
Tertiary amines are added to the reaction mixture also when mixed anhydrides are generated:



Understandably, production of the same mixed anhydrides is accompanied by less racemization if it is carried out with the help of 1-ethyloxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), because no addition of tertiary base is required and the basicity of the quinoline formed in the reaction is negligible. It is more difficult to counteract the effect of an intramolecular basic center, even if weak, such as the imidazole nucleus in the histidine side chain. While the here shown cyclization and enolization



do not eliminate the ability of the activated species to acylate the amine component (acylimidazoles are good acylating agents) the chiral integrity of the histidine residue may certainly suffer in the process. Racemization via enolization might occur without cyclization as well, particularly because the enol can be stabilized in enolate form:

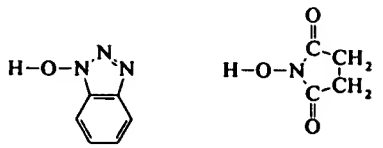


Hence it appears advantageous to further reduce the basic character of the imidazole by blocking, preferably at the π-nitrogen atom.

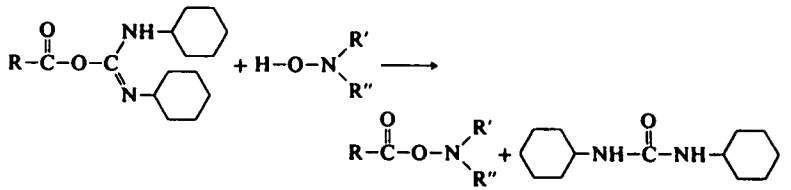
It is not surprising that a process involving proton abstraction is influenced by the polarity of the solvent. Base catalyzed racemization of active esters is fast in polar solvents such as dimethylformamide and slow in non-polar media, for instance in toluene. It is rather unfortunate that such non-polar solvents are more often than not impractical in peptide synthesis. The poor solubility of most blocked intermediates in the commonly used organic solvents severely limits their use and in the preparation of larger peptides indeed dimethylformamide is most frequently applied. The problem of solubility is less serious in solid phase peptide synthesis (cf. Chapter X), where no real solvent is needed but merely a medium in which the polymeric support properly swells. This function is fulfilled by dichloromethane; its effect on racemization lies between the extremes mentioned.

Proton abstraction from the chiral carbon atom can be suppressed by the addition of weakly acidic materials to the reaction mixture. Of the numerous additives tested 1-hydroxybenzotriazole (König and Geiger 1970a) and N-hydroxysuccinimide (Weygand et al. 1966) are routinely used in the practical execution of coupling. These compounds are not acidic enough to protonate the

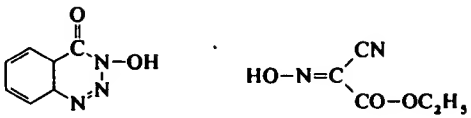
amino group



of the amine component and therefore they do not interfere with its acylation, but their acidity is sufficient to provide competition in abstraction of the proton from the carbon atom of activated intermediates. The significance of these additives is based however, not merely on their acidic character: many other weak acids perform poorly in the role of racemization suppressing agents. Both additives are related to hydroxylamine and function as powerful auxiliary nucleophiles. They react with overactivated intermediates, such as the O-acylisourea in carbodiimide mediated couplings,



reducing thereby the lifetime of the racemization prone species. The active esters produced in these reactions have higher chiral stability. In their reaction with the amine-component the additive is regenerated and assures a continued beneficial effect. Strangely, the highly efficient additives 3-hydroxy-3,4-dihydro-1,2,3-benzotriazine-4-one (König and Geiger 1970b) and 2-hydroximinocyanoacetic acid ethyl ester (Itoh 1973) have not been widely used so far although their effect on the prevention of racemization exceeds that of the popular 1-hydroxybenzotriazole.



The rather general measure that can be taken against side reactions, the use of both the carboxyl-component and the amine-component in high concentration, is applicable for the suppression of racemization as well. However, poor solubility of intermediates, sometimes even in dimethylformamide, presents a formidable obstacle compounded by the high molecular weight of some blocked peptides. Thus a high molar concentration of both components is often

unattainable. On the other hand, if the carboxyl component is not a peptide derivative but rather the blocked and activated form of a single amino acid, it can be used in excess. This excess can be adjusted to provide for a concentration which remains sufficiently high (for instance more than 0.1 molar) throughout the coupling reaction. The relative simplicity of the blocked *and* activated amino acid derivatives and their availability from commercial sources render this sacrifice usually acceptable. Over and above the possibility of performing coupling reactions according to the *principle of excess*, addition of single amino acid residues has a further important benefit in the conservation of chiral purity: the most commonly used amine-protecting groups, the benzyloxycarbonyl and the tert.butyloxycarbonyl group, efficiently prevent racemization in most cases. As mentioned before, this is a common feature of urethane-type blocking groups and applies for the acid-stable, base-sensitive 9-fluorenylmethyloxycarbonyl (Fmoc) group as well.

In the preceding discussion we have dealt only with racemization during peptide bond formation. Loss of chiral purity can occur, however, also during certain processes of deprotection. Hydrogenolysis is quite innocuous in this respect and acidolysis is harmful only if it is carried out under drastic conditions, such as elevated temperature. Saponification of esters with alkali can cause measurable racemization. This must be kept at a minimum by carrying out the reaction at ice-bath temperature, preferably at constant p_H. Large excess of alkali certainly must be avoided. The presence of Cu^{++} ions prevents racemization in alkaline hydrolysis (and probably also in many instances of coupling), but complete removal of the metal ions from the complex is not always straightforward.

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IX. Design of Schemes for Peptide Synthesis

In the strategical planning that must precede the synthesis of a larger peptide, racemization is one of the most important considerations. Therefore, it seems to be appropriate to discuss the various schemes of synthesis at this point. Due to the individuality of amino acid residues and to variations in the properties of blocked intermediates, it appears impractical to propose a general scheme (strategy) that would be applicable for any peptide. Peptide synthesis should be based on retrosynthetic analysis, starting with identification of the problems inherent in the sequence of the target compound.

In principle three approaches are possible: A. condensation of peptide segments; B. stepwise chain-building starting with the N-terminal residue; and C. stepwise chain building starting at the C-terminus. We will attempt to evaluate these alternatives, but with some reservation: there is no consensus among peptide chemists in this area.

A. Segment Condensation

In the earliest days of practical peptide synthesis, in the preparation of the nonapeptide oxytocin or the octapeptide angiotensin, segment condensation appeared to be the obvious strategy. Reduction of a major task to smaller problems, a Cartesian approach, is clearly attractive. Equally important is, however, the possibility of dividing the effort among members of a team. Preparation of the individual segments, often dipeptides, could be entrusted to less experienced coworkers while the arduous task of segment condensation needs an adept in peptide chemistry. A similar distribution of responsibilities is not feasible in stepwise chain lengthening. These considerations must have guided the investigators who undertook the synthesis of biologically active peptides in the nineteen fifties. The retrosynthetic scheme for the synthesis of the octapeptide angiotensin is shown here as an example: